

**Regulation of intra-adipose cortisol concentrations**  
*in vivo* in humans

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## Abstract

Intra-adipose cortisol is derived from the systemic circulation via the hypothalamic-pituitary-adrenal axis (HPAA) and generated locally through conversion of inactive cortisone to cortisol by the intra-cellular enzyme 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1). This thesis addresses the relative contributions of the HPAA and adipose tissue 11 $\beta$ HSD1 to the adipose tissue glucocorticoid pool and describes development and validation of a novel stable isotope tracer, 1,2 [ $^2\text{H}$ ]<sub>2</sub>-cortisone (d2-cortisone), to measure 11 $\beta$ HSD1-dehydrogenase activity in adipose tissue and skeletal muscle *in vivo*.

In otherwise healthy females (n=6) undergoing hysterectomy for a benign indication, an intravenous infusion of d4-cortisol was administered and subcutaneous and omental adipose tissue biopsies were obtained along with concomitant peripheral venous blood, to measure the rate of exchange of cortisol between plasma and adipose tissue for comparison with rates of intra-cellular cortisol generation by 11 $\beta$ HSD1. Cortisol concentrations and enrichment with d4-cortisol were lower in adipose tissue than in plasma. The rate of accumulation of d4-cortisol in adipose tissue depots was ~0.5nmol/kg/h despite the infusion contributing 1.9 $\mu$ mol/h d4-cortisol into the circulation, and the proportion of the intra-adipose cortisol pool replaced each hour was ~10%. The contribution of 11 $\beta$ HSD1 to this turnover could not be quantified since very little substrate d3-cortisone accumulated in adipose during infusion.

Method development for d2-cortisone included optimising LC-MS/MS conditions, confirming that d2-cortisone was a substrate for human 11 $\beta$ HSD1 and that no significant primary isotope effect existed. The pharmacokinetics of d2-cortisone were assessed *in vivo* in healthy male volunteers (n=3). The method was validated by measuring whole body cortisone production in healthy volunteers (n=3) before and after eating liquorice which

resulted in a ~50% fall in cortisone production. 11 $\beta$ HSD1-dehydrogenase activity was measured in adipose tissue and skeletal muscle in healthy volunteers (n=6) using d2-cortisone and substantial 11 $\beta$ -dehydrogenase activity was present in both tissues (~1.5-fold higher 11 $\beta$ -dehydrogenase activity than 11 $\beta$ -reductase activity in adipose tissue and approximately equal 11 $\beta$ -reductase and 11 $\beta$ -dehydrogenase activity in skeletal muscle). 11 $\beta$ HSD1-reductase activity was also assessed using a 9,11,12,12 [<sup>2</sup>H]<sub>4</sub>-cortisol infusion (d4-cortisol). Skeletal muscle and adipose tissue displayed 11 $\beta$ -reductase activity. In adipose tissue this activity was of a similar magnitude to previous reports. Insulin increased whole body 11 $\beta$ -reductase activity, but did not switch 11 $\beta$ HSD1 direction in muscle or adipose tissue, indicating the predominant effect of insulin may be on hepatic 11 $\beta$ HSD1.

Therefore, turnover of the intra-adipose tissue glucocorticoid pool is slow and it is unlikely that rapid acute fluctuations in circulating cortisol are reflected in adipose tissue, although this has not been confirmed under normal physiological conditions. Secondly, 11 $\beta$ HSD1 may be bidirectional in human subcutaneous adipose tissue and skeletal muscle *in vivo*, and insulin does not regulate the balance of activities. However, in this study blood sampling occurred from blood vessels which express 11 $\beta$ HSD2, and thus some of the measured dehydrogenase activity in this study may reflect endothelial 11 $\beta$ HSD2 activity. Together these findings further our understanding of adipose tissue cortisol physiology in health, suggesting that 11 $\beta$ HSD1 may play a relatively important role in modulating activation of glucocorticoid receptors in adipose tissue, and that dysregulation or inhibition of 11 $\beta$ HSD1 may affect cortisol inactivation as well as regeneration.

## Declaration

I declare that this thesis was written by myself and the work presented is my own, with the exceptions listed below:

- i. *Cannulation of vessels and calculation of adipose blood flow for the Oxford arterio-venous sampling study was undertaken by Dr Konstantinos Manolopoulos, Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital, Oxford.*
- ii. *Extraction of urinary glucocorticoids for the LICORT study was performed by Mrs Alison Rutter, Endocrinology Unit, University of Edinburgh.*
- iii. *FT-MS of deuterated steroids was undertaken by Drs RuAngelie Edreda-Ebel and Dave Watson, Strathclyde Institute for Pharmacy and Biomedical Science, University of Strathclyde.*
- iv. *Collection of adipose biopsies was undertaken by surgical teams in the gynaecology theatres, Royal Infirmary of Edinburgh.*

I declare that this work has not been submitted for any other degree.

---

Katherine Ann Hughes, Edinburgh, July 2010.

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## List of Abbreviations

<i>Abbreviation</i>	
ACRD	Apparent cortisone reductase deficiency
ACTH	Adrenocorticotrophic hormone
ASV	Adipose stromal vascular cells
BMI	Body mass index
BSA	Bovine serum albumin
CBG	Cortisol binding globulin
C/EBP	CCAAT/enhancer binding protein
CBX	Carbenoxolone
CID	Collision induced dissociation
CRH	Corticotrophin releasing hormone
CYP	Cytochrome P450 enzymes
D	Deuterium
DHEA	Dehydroepiandrosterone
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
E	Cortisone
EI	Electron impact
ER	Endoplasmic reticulum
F	Cortisol
FBF	Forearm blood flow
FCS	Fetal calf serum
FT-MS	Fourier-transform mass spectrometry
G-6-P	Glucose-6-phosphate
G6P-ase	Glucose-6-phosphatase

GC-MS	Gas chromatography mass spectrometry
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GT	Glucose transporter
H6PDH	Hexose-6-phosphate dehydrogenase
HDL	High density lipoprotein
HEK293	Human embryonic kidney cell line 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPAA	Hypothalamic-pituitary-adrenal axis
HPLC	High performance liquid chromatography
HSD	Hydroxysteroid dehydrogenase
IGF-1	Insulin-like growth factor-1
IL	Interleukin
LDL	Low density lipoprotein
LC-MS	Liquid chromatography mass spectrometry
MDR	Multi-drug resistance
MO-TMS	Methoxime-trimethylsilyl
MR	Mineralocorticoid receptor
M/Z	Mass charge ratio
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
OAT3	Organic-ion transporter
OFN	Oxygen free nitrogen
6PG	6-Phosphogluconolactone
PEPCK	Phosphoenolpyruvate carboxykinase
RaE	Rate of appearance of cortisone

RaF	Rate of appearance of cortisol
Rad3F	Rate of appearance of d3-cortisol
RME	Relative mean error
RSD	Relative standard deviation
SAME	Syndrome of apparent mineralocorticoid excess
SDRs	Short chain dehydrogenase / reductase family
SEM	Standard error of the mean
SS	Steady state
THE	Tetrahydrocortisone
THF	Tetrahydrocortisol
TTR	Tracer: tracee ratio
TNF $\alpha$	Tissue necrosis factor alpha
V/V	Volume for volume
W/V	Weight for volume

## List of publications, oral presentations and awards

### Review

- **KA Hughes**, SP Webster, BR Walker. The Therapeutic Applications of 11-Beta Hydroxysteroid Dehydrogenase Type 1 (11 $\beta$ -HSD1) Inhibitors in Type 2 Diabetes and Obesity. Expert Opinion on Investigational Drugs. 2008; 17(4):481-96.

### Abstracts

- **KA Hughes**, RM Reynolds, R Andrew, BR Walker. Measuring cortisone production in man using a new stable isotope tracer. Scottish Society for Experimental Medicine, Edinburgh, UK 2008.
- **KA Hughes**, RM Reynolds, R Andrew, BR Walker. Measuring cortisone production in man using a new stable isotope tracer. British Endocrine Societies, Harrogate, UK, 2009.
- **KA Hughes**, RM Reynolds, HOD Critchley, R Andrew, BR Walker. Glucocorticoids turn over slowly in human adipose tissue *in vivo*. Annual meeting of the Endocrine Society (ENDO), San Diego, 2010.
- **KA Hughes**, K Manolopoulos, R Andrew, F Karpe & BR Walker. Reversible interconversion of cortisol and cortisone in human subcutaneous adipose tissue and skeletal muscle *in vivo*. Annual meeting of the Endocrine Society (ENDO), San Diego, 2010.

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- Caledonian Endocrine Society, Peebles, UK, 2008.
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## Original research accepted for publication

- **KA Hughes**, RM Reynolds, R Andrew, HOD Critchley, Brian R Walker. Glucocorticoids turn over slowly in human adipose tissue *in vivo*.  
*Accepted as an original article in the Journal of Clinical Endocrinology and Metabolism.*

## Manuscripts in preparation

- **KA Hughes**, R Andrew, S Denham, R Edrada-Ebel, DG Watson, RM Reynolds, BR Walker. Quantifying cortisone production in man using deuterated cortisone.
- **KA Hughes**, KN Manolopoulos, R Andrew, F Karpe and BR Walker. Interconversion of cortisol and cortisone in human subcutaneous adipose tissue and skeletal muscle in vivo: not such futile cycling.

# **Chapter 1**

## **Introduction**

## **1.0 Introduction**

Glucocorticoids, such as cortisol, have a number of physiological functions including homeostatic regulation of carbohydrate, amino acid, lipid metabolism, blood pressure regulation and modulation of the immune response. It is crucial that cortisol levels are elevated in times of acute physical stress such as sepsis or physical trauma, but if cortisol elevations are sustained for prolonged periods in the absence of these stimuli, the homeostatic responses become maladaptive and the features of Cushing's syndrome result, including central obesity and hyperglycaemia. As some of the Cushing's syndrome phenotype is largely reversible upon removal of glucocorticoid excess, the potential of modulating glucocorticoid action was conceived as a therapeutic target in diabetes and obesity. However, antagonising glucocorticoids systemically presents the risk of inducing adrenal insufficiency at times of acute stress. Therefore strategies have been developed to modulate glucocorticoid action specifically in target tissues to circumvent this side effect. One particular drug target is the intracellular enzyme 11 $\beta$ -hydroxysteroid dehydrogenase type 1 which is expressed in many tissues including adipose. To develop an effective treatment, glucocorticoid physiology in the target tissues need to be fully understood. Therefore, this thesis will focus on regulation of glucocorticoid metabolism in adipose tissue and novel approaches to measure adipose glucocorticoids *in vivo*.

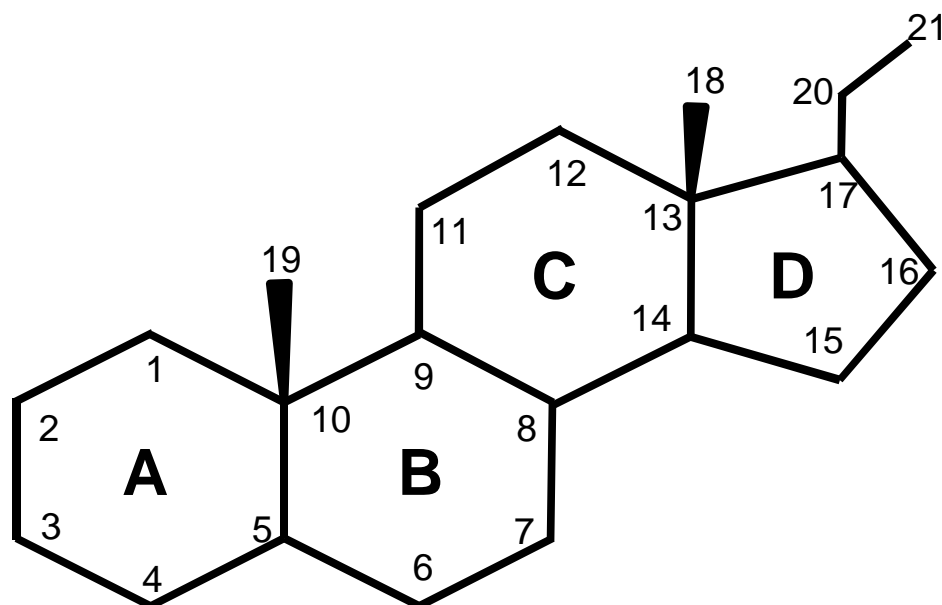
## **1.1 Glucocorticoids**

### **1.1.1 Hormone structure, synthesis, transport and metabolism**

Glucocorticoids (predominantly cortisol in man and corticosterone in rodents) are synthesised *de novo* from the common precursor cholesterol in the adrenal gland. All steroid



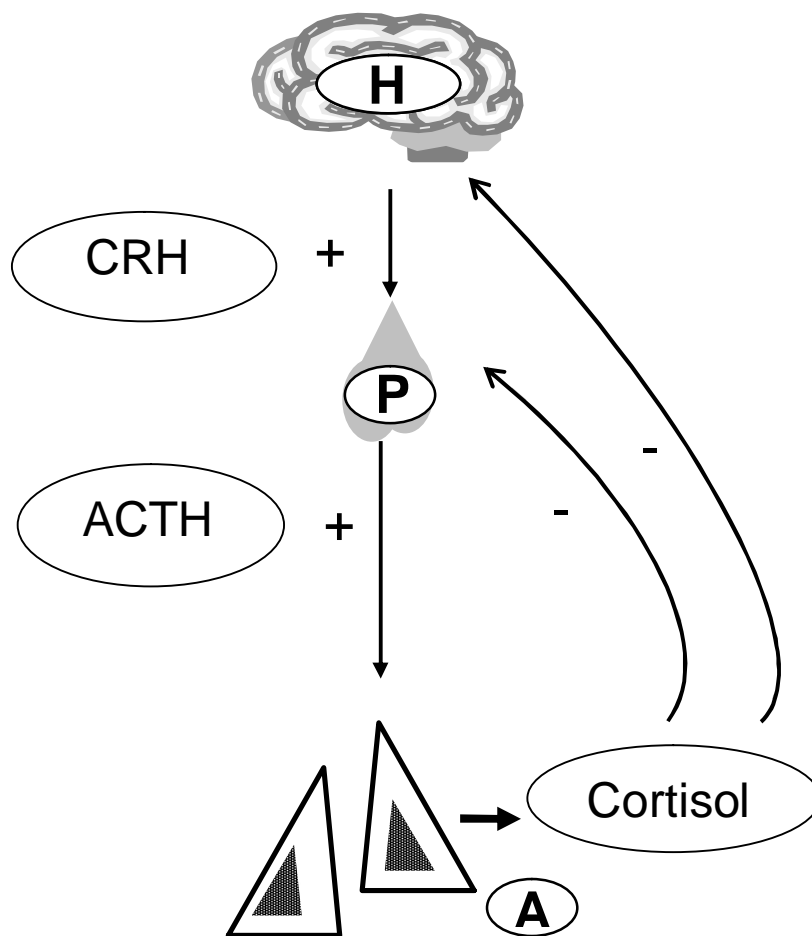
hormones have a similar structure comprising a cyclopentane ring and three cyclohexane rings; the properties of each individual steroid are determined by the presence of different chemical groups at specific positions on the molecule (Figure 1.1). Glucocorticoids are mainly synthesised in the zona fasciculata of the adrenal cortex, catalysed by a series of cytochrome P450 (CYP) enzymes, which reside in the membranes of the endoplasmic reticulum and mitochondria. There is some glucocorticoid synthesis in the zona reticularis also. Glucocorticoids are not stored in the adrenal gland but are synthesised *de novo* and released when required.



**Figure 1.1 Basic steroid structure**

The basic steroid ring structure comprising a cyclopentane ring and three cyclohexane rings. Conventional labelling identifies the four carbon ring structures by letters, and the individual carbon atoms by numbers. Chemical groups are designated according to the number of the carbon atom to which they are attached.

Glucocorticoid synthesis and release is regulated by neuro-endocrine signals such as physical stress and pro-inflammatory cytokines released during inflammation. Both  $\text{TNF}\alpha$  and  $\text{IL-1}\beta$  stimulate glucocorticoid production by enhancing expression of both CRH and ACTH, which modulate the hypothalamic-pituitary-adrenal axis (Munck *et al*, 1984; Turnbull & Rivier, 1999). Glucocorticoids negatively feedback to the hypothalamic-pituitary-adrenal axis to maintain physiological circulating glucocorticoid levels (Figure 1.2).



**Figure 1.2 The hypothalamic-pituitary-adrenal axis**

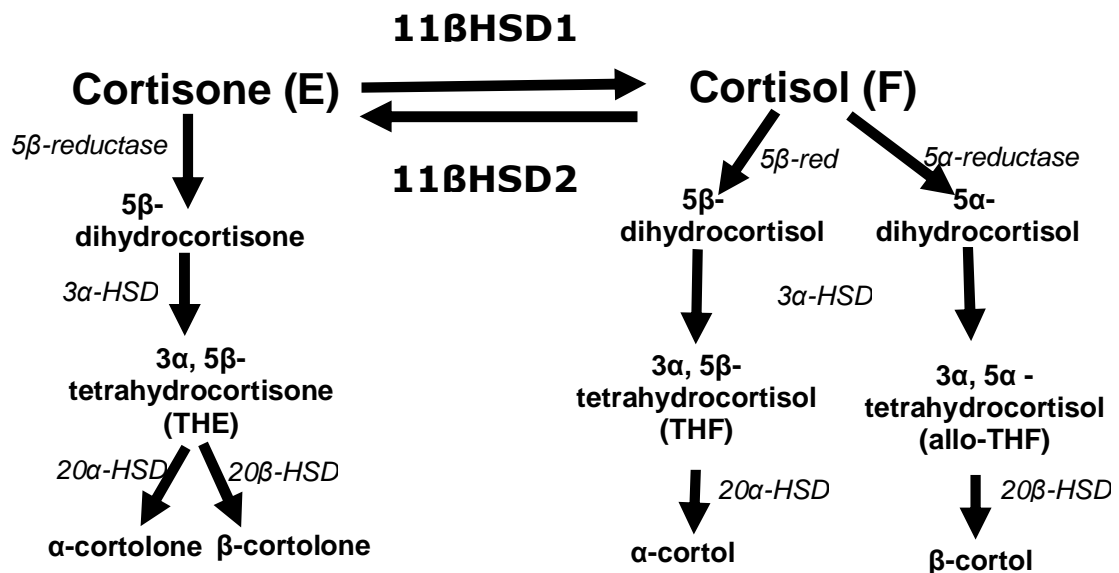
Corticotrophin releasing hormone (CRH) is released from the hypothalamus (H) and stimulates the anterior pituitary (P) to release adrenocorticotrophic hormone (ACTH). ACTH stimulates the adrenal cortex (A) to produce cortisol, which in turn provides negative feedback control on CRH and ACTH to maintain physiological circulating glucocorticoid levels.

As a result of diurnal changes in ACTH pulse frequency and amplitude, glucocorticoid synthesis is subject to diurnal variation, with plasma levels highest just prior to waking and falling prior to sleep (Dallman *et al*, 1993). In addition to a diurnal rhythm, glucocorticoids are also secreted in an ultradian pattern which affects transcriptional regulation *in vivo* and in cells lines (Stavreva *et al*, 2009). This may serve to modulate the response to the glucocorticoid receptor activation (Stavreva *et al*, 2009).

Plasma glucocorticoids are largely bound to corticosteroid-binding globulin (CBG) and albumin, with only 5-10% circulating in the free unbound state (Hammond *et al*, 1990). These binding proteins act as a buffer as only free steroids are able to cross cell membranes to bind to the intracellular glucocorticoid receptors, and affect gene transcription. At high glucocorticoid concentrations eg prior to waking in the morning, binding proteins may become saturated, which may amplify the normal diurnal variations in circulating levels of free glucocorticoids.

Traditionally, glucocorticoid transport across the cell membrane was assumed to be a passive process following a concentration gradient. However, an increasing number of transporters of steroids have been identified, such as the organic anion transporter-3 (OAT3) in the adrenal (Asif *et al*, 2005), the multi-drug resistance (MDR) P-glycoprotein in the central nervous system (Karssen *et al*, 2001), and the glucocorticoid importer in the liver (Lackner *et al*, 1998). Moreover, these mechanisms can differentially allow transport of specific steroids into tissues, for example in the mouse central nervous system corticosterone can enter the tissues freely, whilst the MDR1 glycoprotein membrane pump reduces access of infused [<sup>3</sup>H]-cortisol (Karssen *et al*, 2001).

Inactivation of glucocorticoids involves conversion to inactive metabolites, which predominantly occurs in the liver. However, the kidney also displays some catabolic activity. Glucocorticoids are then renally excreted (Figure 1.3).



**Figure 1.3 Metabolism of cortisol and cortisone**

Cortisol and inert cortisone are interconverted by the isozymes of 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ HSD). The steroid A-ring is reduced across the 4-5 double bond by 5 $\alpha$ / $\beta$ -reductases to form the dihydro-product. Glucocorticoids reduced at the 5 -position are substrates for 3 $\alpha$ -hydroxysteroid dehydrogenases (3 $\alpha$ HSD), which are further reduced by the 20 $\alpha$ / $\beta$ -hydroxysteroid dehydrogenases to form cortols and cortolones. Glucocorticoid metabolites are transformed into hydrophilic molecules (the process of conjugation) to ensure solubility in aqueous biological fluids prior to excretion. The two major pathways of conjugation include the formation of glucuronides through uridine diphosphoglucuronic acid (UDPGA) and a glucuronyl transferase and the formation of sulphates, catalysed by sulphokinases.

## 1.2 Glucocorticoid action

Glucocorticoids act predominantly through binding to intracellular receptors. There are two receptor subtypes: mineralocorticoid (MR) and glucocorticoid (GR) receptors. GR are widespread whereas MR are found in aldosterone-sensitive tissues such as the distal convoluted tubule, loop of Henle, and collecting tubules of the kidney and the absorptive epithelia of duodenum, jejunum, ileum, colon (Hirasawa *et al*, 1997), the hippocampus and heart (Funder, 2005). Glucocorticoids are able to bind to both GR and MR with equal affinity, however pre-receptor metabolism of glucocorticoids by the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (please see section 1.6.1) prevents illicit occupation of MR in aldosterone-sensitive tissues.

Once inside the cell, glucocorticoid binding leads to dissociation of GR from inhibitory heat shock proteins, GR phosphorylation and activation, dimerisation and translocation to the nucleus where the GR complex binds to specific palindromic DNA motifs called glucocorticoid response elements (GREs). GREs are located in the promoter region of target genes (Yamamoto, 1985) and activation leads to transcription of responsive genes, a process called transactivation. Negative regulation of transcription, transrepression, can also occur through GR binding to negative GREs. Activated GR may also indirectly influence gene transcription through interactions with other transcription factors, such as the signal transducer and activator of transcription-5 (Stat5). It has been demonstrated that Stat5 and GR form a molecular complex which cooperates in the induction of transcription of milk protein genes in mammary epithelial cells (Stoecklin *et al*, 1997).

## 1.3 Physiological effects of glucocorticoids

### 1.3.1 Effects on metabolism

Glucocorticoids have many metabolic effects. Regarding insulin and glucose homeostasis, glucocorticoids impair insulin-dependent glucose uptake in the periphery eg skeletal muscle, enhance gluconeogenesis in the liver (Rooney *et al*, 1994), and inhibit insulin secretion from pancreatic  $\beta$ -cells (Delaunay *et al*, 1997). Glucocorticoids also alter lipid metabolism by stimulating adipocyte lipolysis, which releases free fatty acids into the circulation. In adipose tissue, glucocorticoids induce expression of hormone sensitive lipase (Slavin *et al*, 1994), which hydrolyses diacylglycerides in adipocytes (reviewed in (Macfarlane *et al*, 2008)).

In Cushing's syndrome glucocorticoid excess is associated with marked skeletal muscle atrophy and proximal myopathy. This reduction in skeletal muscle is as a result of reduced protein synthesis and increased protein catabolism. Specifically, synthesis is inhibited by reducing amino acid transport into muscle and abolishing the anabolic effects of insulin and IGF-1 (reviewed in (Schakman *et al*, 2008)). Proteolytic systems such as the ubiquitin-proteasome system, which degrade insulin signalling machinery, are also activated by glucocorticoids (Morgan *et al*, 2009; reviewed in (Schakman *et al*, 2008)).

Glucocorticoid excess can also induce osteoporosis, by inhibiting intestinal calcium absorption, increasing renal calcium excretion and by inhibiting new bone formation (Canalis & Delany, 2002). Glucocorticoids have adverse effects on connective tissue through inhibition of fibroblast function (Pratt & Aronow, 1966).

## 1.4 Glucocorticoid action and Metabolic Syndrome

### 1.4.1 Glucocorticoid secretion

The metabolic abnormalities found in central obesity, type 2 diabetes and the metabolic syndrome are similar to those seen in syndromes of prolonged exogenous or endogenous glucocorticoid excess (Cushing's syndrome). Bjorntorp *et al* (Bjorntorp *et al*, 1999) proposed that subtle alterations in the hypothalamic-pituitary-adrenal axis (HPA) and cortisol homeostasis may provide the link between the cause and metabolic consequences of central obesity. The metabolic syndrome refers to a clustering of metabolic abnormalities that are associated with an increased risk of type 2 diabetes and cardiovascular disease (Balkau & Charles, 1999). The principal features are insulin resistance, central abdominal obesity, abnormalities of glucose and lipid metabolism, hypertension, and a pro-inflammatory state.

Cross-sectional and case-control studies have shown higher plasma, urinary or salivary cortisol levels in association with some components of the Metabolic Syndrome, including hypertension, insulin resistance, glucose intolerance, and in older men with type 2 diabetes (Filipovsky *et al*, 1996; Litchfield *et al*, 1998; Liu *et al*, 2005; Phillips *et al*, 1998; Reynolds *et al*, 2001; Reynolds *et al*, 2001b; Stolk *et al*, 1996; Walker *et al*, 1998; Walker *et al*, 2000). Moreover, long-term exogenous glucocorticoid excess is associated with increased risk of cardiovascular events (Souverain *et al*, 2004; Wei *et al*, 2004). However, in idiopathic obesity, although cortisol production rate is increased, this may be as a consequence of enhanced metabolic clearance of cortisol (Walker, 2006; Lottenberg SA *et al*, 1998) or impaired HPA-axis negative feedback (Mattsson *et al*, 2009) and circulating cortisol levels are not elevated (Ljung *et al*, 1996; Phillips *et al*, 1998; Walker *et al*, 2000).

## 1.5 Tissue responses to glucocorticoids

Until recently, tissue glucocorticoid levels were thought to be exclusively determined by plasma levels of free glucocorticoids and tissue responses to glucocorticoids controlled by the density and availability of glucocorticoid receptors. It is now established that the biological effects of cortisol are controlled not only by circulating hormone levels via the HPA axis, but at the tissue level by pre-receptor enzymes that either limit or amplify access of ligands to glucocorticoid receptors. There is some evidence of GR dysfunction in Metabolic Syndrome (van Rossum *et al*, 2004; Walker, 2007), perhaps with a genetic basis in type 2 diabetes and hypertension (Franks *et al* 2004; Nair *et al* 2004; Walker, 2006). However, from a therapeutic perspective, the pre-receptor enzymes have attracted more attention. The presence of an enzyme in some target tissues which amplifies local cortisol concentrations provides an opportunity for development of enzyme inhibitors selectively to reduce glucocorticoid action in specific sites.

## 1.6 11 $\beta$ -Hydroxysteroid dehydrogenases

Intra-cellular glucocorticoid levels are influenced in many tissues by the two isozymes of 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ HSD).

### 1.6.1 11 $\beta$ - Hydroxysteroid dehydrogenase type 2

11 $\beta$ HSD type 2 is found in aldosterone sensitive tissues such as the distal nephron, colon and placenta (Edwards *et al* 1988; Stewart *et al* 1987) and functions to rapidly inactivate cortisol (corticosterone in rats and mice) and reserve MR activation specifically for aldosterone (Figure 1.4) (Funder *et al*, 1988; Edwards *et al* 1988). In the hippocampus however, MR is



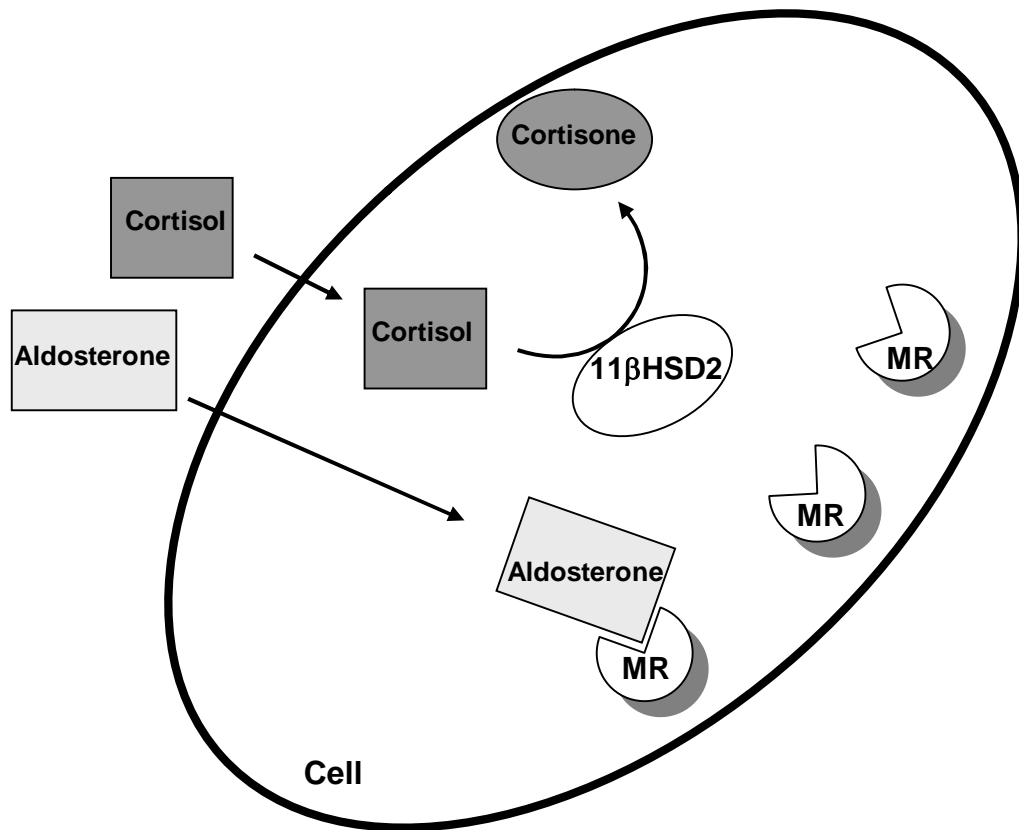
not protected by 11 $\beta$ HSD2 and is activated by both glucocorticoids and mineralocorticoids (Sheppard & Funder, 1987).

The gene for 11 $\beta$ HSD2, located on chromosome 16 in humans and chromosome 8 in mice, has 77% sequence homology between the 2 species (White, 2001). 11 $\beta$ HSD2 is a microsomal membrane-bound enzyme which uses nicotinamide adenine dinucleotide (NAD) as its cofactor and has a  $K_m$  for cortisol in the nanomolar range (Brown *et al*, 1993). Congenital deficiency of 11 $\beta$ HSD2 (Dave-Sharma *et al*, 1998), or transgenic deletion (Kotelevtsev *et al*, 1999) in mice, produces the syndrome of apparent mineralocorticoid excess with a phenotype of hypertension and sodium retention due to activation of MR by cortisol. Similar abnormalities result from consumption of large quantities of liquorice (Epstein *et al*, 1977), which contains inhibitors of 11 $\beta$ HSD2 (Stewart *et al*, 1987). More subtle 11 $\beta$ HSD2 deficiency may be important in essential hypertension (Ferrari *et al*, 1996; Walker *et al*, 1993).

Mice with adipose tissue specific over-expression of human 11 $\beta$ HSD2 (under the control of the murine aP2 promoter; aP2-h11 $\beta$ HSD2) (Kershaw *et al*, 2005) resist weight gain on high-fat diet and have improved glucose tolerance and insulin sensitivity. aP2-h11 $\beta$ HSD2 Mice also have a favourable adipocytokine profile, suggesting that inactivation of glucocorticoids specifically in adipose tissue is an important determinant of a favourable metabolic phenotype.

Although initial studies failed to demonstrate any significant 11 $\beta$ HSD2 in adipose tissue (Bujalaska *et al*, 2002), more recent studies have shown 11 $\beta$ HSD2 expression in both rat adipocytes and adipose stromal vascular (ASV) cells (Milagro *et al*, 2007), in subcutaneous

adipocytes of obese women (albeit at much lower levels than 11 $\beta$ HSD1) (Engeli *et al*, 2004), and the stromal fraction of human omental adipose tissue (Lee *et al*, 2008).



**Figure 1.4 Pre-receptor metabolism of active glucocorticoids by 11βHSD2**

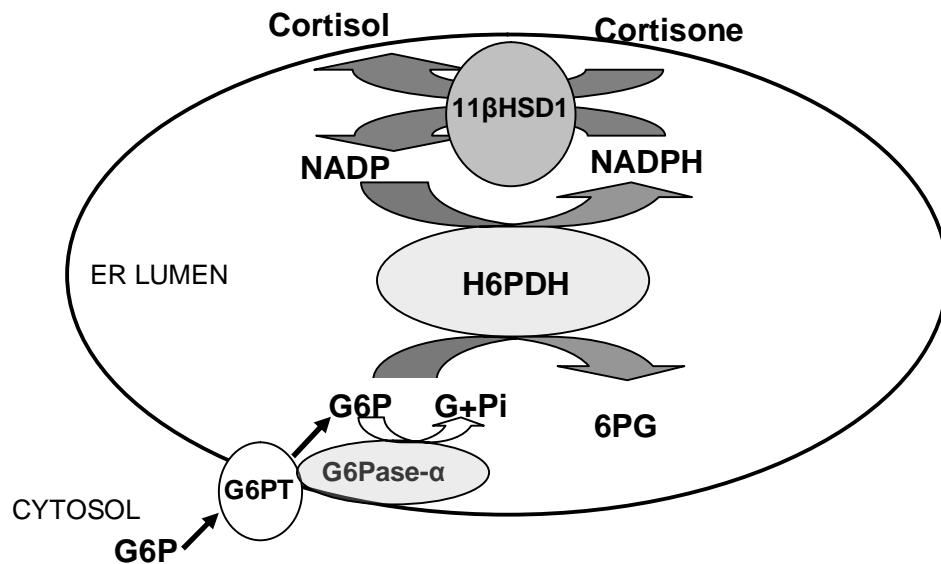
Active glucocorticoids (cortisol, corticosterone) are inactivated by the type 2 isozyme of 11β-hydroxysteroid dehydrogenase, thus preventing illicit occupation of the MR by glucocorticoids and conferring aldosterone specificity on the receptor.

### 1.6.2 11 $\beta$ - Hydroxysteroid dehydrogenase type 1

11 $\beta$ HSD type 1 is a low affinity NADPH-dependent microsomal (endoplasmic reticulum) enzyme that belongs to the short chain alcohol dehydrogenase / reductase family (SDRs). The gene for 11 $\beta$ HSD1 is located on chromosome 1 in both humans and mice, and is highly conserved between species, with ~80% sequence homology (Tomlinson *et al*, 2004). In early purification studies, 11 $\beta$ HSD1 activity showed bi-directionality *in vitro* (Lakshmi & Monder, 1988). Further studies have demonstrated that the predominant reaction direction in intact cells *in vitro* and *in vivo* is reductase (Seckl & Walker, 2001), converting cortisone to cortisol, driven by co-localisation within the lumen of the endoplasmic reticulum of the NADPH-generating enzyme hexose-6-phosphate dehydrogenase (H6PDH) as it catalyses the first step in the pentose phosphate pathway (Hewitt *et al*, 2005) (Figure 1.5).

11 $\beta$ HSD1 is highly expressed in the liver (Lakshmi & Monder, 1988) and in adipose tissue (Bujalska *et al*, 1997) although it is also found in a number of other tissues, including brain (Sandeep *et al*, 2004), blood vessel wall (Small *et al*, 2005), macrophages (Gilmour *et al*, 2006), eye (Rauz *et al*, 2003), bone (Eijken *et al*, 2005), lung (Hundertmark *et al*, 2002), and ovary (Thomas *et al*, 1998).

Congenital 11 $\beta$ HSD1 deficiency or apparent cortisone reductase deficiency (ACRD) is characterised by failure to regenerate cortisol from inert cortisone, resulting in increased cortisol clearance, compensatory activation of the HPA axis, and androgen excess mediated through the ACTH drive. Clinically, ACRD is characterised by hirsutism, oligomenorrhoea and sub-fertility. Inactivating mutations in the H6PDH gene which prevent 11 $\beta$ HSD1-reductase generation of cortisol have been reported as a cause of ACRD (Lavery *et al*, 2008).



**Figure 1.5: Relationship between hexose-6-phosphate dehydrogenase (H6PDH) and 11βHSD1 *in vivo*.**

11βHSD1 is co-localised with H6PDH within the lumen of the endoplasmic reticulum. H6PDH generates NADPH cofactor for the reductase reaction of 11βHSD1 as it catalyses the first step in the pentose phosphate pathway. In this pathway, glucose-6-phosphate (G6P) enters the ER lumen via the glucose-6-phosphate transporter (G6PT) and is a substrate for glucose-6-phosphatase-α and undergoes hydrolysis to form glucose (G) and inorganic phosphate (Pi), or is utilised by H6PDH. H6PDH converts G6P to 6-phosphogluconolactonate (6PG) and generates NADPH for 11βHSD1. Adapted from (Tomlinson *et al*, 2004) and (Walker *et al* 2007).

## 1.7 11 $\beta$ HSD1 in adipose tissue

Human adipose tissue contains a glucocorticoid pool derived from both the systemic circulation and from local regeneration of cortisol by 11 $\beta$ HSD1. Concentrations of glucocorticoids in human subcutaneous adipose tissue biopsies have been estimated using radio-immunoassays (RIAs) and liquid chromatography tandem mass spectrometry (LC-MS/MS) with contrasting results (Wake *et al*, 2003; Lindsay *et al*, 2003; Ronquist-Nii & Edlund, 2005). Using highly specific and sensitive LC-MS/MS, adipose cortisol has been reported at concentrations of  $12.4 \pm 0.6$  and cortisone at  $3.0 \pm 0.3$  nmol/kg (Ronquist-Nii & Edlund, 2005), whereas cortisol concentrations ranging from 16.0- 157.0 nmol/kg have been reported using RIAs in subjects with a range of BMIs. Using *in vivo* microdialysis, subcutaneous adipose interstitial fluid cortisol concentrations have been reported as  $\sim 7$  nmol/L (Tomlinson *et al*, 2007).

*Ex-vivo*, 11 $\beta$ HSD1 is 'set' towards generation of cortisol in human omental adipocytes (Bujalska *et al*, 2002) and omental stromal vascular cells (SVCs) when stimulated with insulin and cortisol (Bujalska *et al*, 1997). The reductase reaction also predominates in murine SVCs from mesenteric and subcutaneous depots (De Sousa Peixoto *et al*, 2008). In both species visceral cells display greater reductase activity than subcutaneous cells (De Sousa Peixoto *et al*, 2008; Bujalska *et al*, 1997). *In vivo*, significant release of cortisol from subcutaneous adipose tissue (Stimson *et al*, 2009) has also been described.

*Ex-vivo* and *in vivo* studies have also demonstrated subcutaneous and omental SVC and subcutaneous whole adipose tissue dehydrogenase activity in primary cell culture experiments and in subcutaneous adipose microdialysis studies (Bujalska *et al*, 2002; Wake *et al*, 2006). In states of NADPH deficiency eg when 11 $\beta$ HSD1 is liberated from the

microsomal environment or when H6PDH is lost through transgenic manipulations in mice (Lavery *et al*, 2006), 11 $\beta$ HSD1-dehydrogenase activity predominates. As some studies have shown that adipose tissue 11 $\beta$ HSD2 expression is very low / negligible (Lee *et al*, 2008; Svendsen *et al*, 2009) and 11 $\beta$ HSD1 expression is abundant (Bujalska *et al*, 2002), human adipose may have 11 $\beta$ HSD1-dehydrogenase activity *in vivo*.

However, as acknowledged by the authors of the *in vivo* study (Wake *et al*, 2006), the finding of 11 $\beta$ HSD1-dehydrogenase activity needs to be interpreted with some caution. Firstly, measured dehydrogenase activity may relate to the presence of vascular endothelial 11 $\beta$ HSD2 in adipose tissue (Wake *et al*, 2006). Secondly, the technique of micro-dialysis can cause local tissue trauma upon insertion of the cannulae into the adipose tissue, which may act to increase dehydrogenase activity through causing disruption of the co-localised H6PDH and 11 $\beta$ HSD1 and thus diminishing co-factor availability. Therefore, the question of whether 11 $\beta$ HSD1-dehydrogenase activity occurs in human adipose tissue *in vivo* remains controversial.

The issue of adipose tissue possessing some dehydrogenase activity *in vivo* remains a priority that needs to be assessed for the development of new 11 $\beta$ HSD1 inhibitor drugs. These agents exert their effects by inhibiting 11 $\beta$ HSD1-reductase and thus lowering intracellular glucocorticoids. If 11 $\beta$ HSD1 functions as a dehydrogenase, inactivating glucocorticoids, an 11 $\beta$ HSD1 inhibitor might actually increase, rather than decrease, intracellular cortisol concentrations, depending on the balance of activities. So, although at present there is no convincing evidence of *in vivo* dehydrogenase activity attributable to 11 $\beta$ HSD1 in circumstances other than complete deficiency of H6PDH, further studies are required.

## 1.8 Regulation of 11 $\beta$ HSD1 in adipose

11 $\beta$ HSD1 is highly transcriptionally regulated. Factors known to increase 11 $\beta$ HSD1 expression include glucocorticoids, peroxisome proliferator-activated receptor- $\gamma$  agonists, and some proinflammatory cytokines (TNF $\alpha$ , IL-1 $\beta$ ) (Tomlinson *et al*, 2004). Growth hormone (acting via IGF-I) and liver X receptor agonists inhibit 11 $\beta$ HSD1 expression (Tomlinson *et al*, 2004). The effect of other factors such as sex steroids, insulin, and thyroid hormone (T3) vary between tissues and species studied (Tomlinson *et al*, 2004).

In adipose tissue, the effects of diet, intralipid infusions, PPAR- $\alpha$  and - $\gamma$  agonists and insulin on 11 $\beta$ HSD1 transcription have been assessed, but again the studies show inconsistent regulation depending on the species studied. In both mice (Morton *et al*, 2004b) and rats (Drake *et al*, 2005) a high fat diet decreases 11 $\beta$ HSD1 activity. In humans intravenous intralipid acutely increases subcutaneous adipose 11 $\beta$ HSD1 activity (Wake *et al*, 2006), but longer term treatment with an oral high fat- low carbohydrate diet or medium fat- medium carbohydrate diet does not affect subcutaneous adipose 11 $\beta$ HSD1 mRNA or activity (Stimson *et al*, 2007).

PPAR- $\alpha$  and - $\gamma$  agonists reduce 11 $\beta$ HSD1 activity in the liver (Hermanowski-Vosatka *et al*, 2000) and adipose tissue (Berger *et al*, 2001) of rodents, but in healthy male volunteers, 7 days of fenofibrate (PPAR- $\alpha$  agonist) failed to have an effect on cortisol secretion. Short term rosiglitazone administration (PPAR - $\gamma$  agonist) did however marginally reduce adipose 11 $\beta$ HSD1-activity (Wake *et al* 2007). Four-five weeks of rosiglitazone had no effect on adipose 11 $\beta$ HSD1, whereas longer term treatment in individuals with type 2 diabetes reduced 11 $\beta$ HSD1 expression in subcutaneous adipose tissue (Wake *et al* 2007) in keeping with the rodent studies.



*In vitro* insulin reduces 11 $\beta$ HSD1 in rodent hepatocytes (Jamieson *et al*, 1995), but does not alter 11 $\beta$ HSD1 expression in adipocytes (Bujalska *et al*, 1999). *In vivo*, acute hyperinsulinaemia in humans has been shown to either increase (Wake *et al*, 2006) or temporarily decrease (Sandeep *et al*, 2005) subcutaneous adipose tissue 11 $\beta$ HSD1 activity in lean subjects. However, hyperinsulinaemia does increase whole body 11 $\beta$ HSD1 activity (Wake *et al*, 2006). Given that hyperinsulinaemia increases glucose flux into cells, we aimed to assess if increasing intracellular glucose availability would increase flux in the pentose phosphate pathway, hypothesising that increased glucose delivery to H6PDH may result in increased NADPH co-factor generation. This may in turn increase 11 $\beta$ HSD1-reductase activity, which is NADPH-dependent, or even switch directionality of 11 $\beta$ HSD1 in adipose tissue. This hypothesis has been examined in Chapter 5.

## **1.9 11 $\beta$ HSD1 in obesity and type 2 diabetes**

Alterations in 11 $\beta$ HSD1 have been documented in both animal models and clinical studies of idiopathic obesity and type 2 diabetes. These changes are tissue-specific:

In most, but not all (Morton *et al*, 2005), obese rodents 11 $\beta$ HSD1 expression and activity is reduced in the liver and increased in adipose tissue (Livingstone *et al*, 2000; Masuzaki *et al*, 2001; Liu *et al*, 2003; Morton *et al*, 2004.). Similar differences have been described in obese humans, in whom there is reduced hepatic (Rask *et al*, 2001; Rask *et al*, 2002; Stewart *et al*, 1999), and increased adipose (Desbriere *et al*, 2006; Engeli *et al*, 2004; Kannisto *et al*, 2004; Lindsay *et al*, 2003; Paulmyer-Lacroix *et al*, 2002; Rask *et al*, 2001; Rask *et al*, 2002; Sandeep *et al*, 2005; Wake *et al*, 2003; Westerbacka *et al*, 2003) 11 $\beta$ HSD1 activity (Table 1.1).

Many of these observations in obesity have been made in biopsied adipose tissue. However, using *in vivo* microdialysis, it has been demonstrated that cortisol generation within subcutaneous adipose tissue is increased in obesity (Sandeep *et al*, 2005). Using stable isotope tracer methods however, splanchnic cortisol generation was not increased in obesity (Basu *et al*, 2005), suggesting that any increase in cortisol production by visceral tissue is compensated for by a decrease in hepatic cortisol production (Walker & Andrew, 2006). Therefore, it is important to make tissue-specific measurements in order to dissect the role of 11 $\beta$ HSD1. The magnitude of the effect of altered 11 $\beta$ HSD1 expression on intra-adipose cortisol concentrations in normal conditions also remains to be determined.

Fewer studies of 11 $\beta$ HSD1 have been conducted in type 2 diabetes (Valsamakis *et al*, 2004). Although hepatic 11 $\beta$ HSD1 activity is mildly impaired, adipose tissue 11 $\beta$ HSD1 activity appears to be normal in lean patients with type 2 diabetes (Andrews *et al*, 2002). In obese type 2 diabetic patients, 11 $\beta$ HSD1 expression in skeletal muscle myotubes is increased, which may contribute to the pathogenesis of insulin resistance (Abdallah *et al*, 2005).

**Table 1.1: Summary of studies examining tissue 11 $\beta$ HSD1 expression and activity in obese rodents and humans.**

Reference	Adipose 11 $\beta$ HSD1 expression or activity	Hepatic 11 $\beta$ HSD1 expression or activity
(Morton <i>et al</i> , 2005)	↓	↑
(Livingstone <i>et al</i> , 2000),	↑	↓
(Rask <i>et al</i> , 2001),	↑	↓
(Rask <i>et al</i> , 2002),	↑	↓
(Westerbacka <i>et al</i> , 2003)	↑	↓
(Masuzaki <i>et al</i> , 2001),	↑	-
(Desbriere <i>et al</i> , 2006),	↑	-
(Engeli <i>et al</i> , 2004),	↑	-
(Lindsay <i>et al</i> , 2003),	↑	-
(Sandeep <i>et al</i> , 2005),	↑	-
(Wake <i>et al</i> , 2003),	↑	-
(Kannisto <i>et al</i> , 2004),	↑	-
(Paulmyer-Lacroix <i>et al</i> , 2002)	↑	-

## 1.10 Transgenic rodent models of altered 11 $\beta$ HSD1

Manipulations of 11 $\beta$ HSD1 in mice have confirmed the potential importance of this enzyme in obesity, type 2 diabetes and the Metabolic Syndrome. Mice over-expressing 11 $\beta$ HSD1 in white adipose tissue under the aP2 promoter have normal serum glucocorticoid levels, as in human obesity (Masuzaki *et al*, 2001). However, intra-adipose corticosterone concentrations are increased and these animals develop a Metabolic Syndrome phenotype, with increased

visceral fat mass, glucose intolerance, insulin resistance, and elevated portal corticosterone and free-fatty acid levels (Masuzaki *et al*, 2001). Since the fold-increase in 11 $\beta$ HSD1 activity in adipose tissue is similar in these mice to the up-regulation observed in adipose tissue in human obesity, these findings suggest that increased intra-adipose 11 $\beta$ HSD1 may be responsible for the metabolic complications of obesity, which when present in humans are associated with increased cardiovascular risk.

Conversely, mice with targeted disruption of the *HSD11B1* gene locus (11 $\beta$ HSD1  $-/-$  mice) resist hyperglycaemia provoked by high fat feeding and, on starvation, show attenuated activation of key hepatic enzymes involved in gluconeogenesis (Glucose-6-Phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (Kotelevtsev *et al*, 1997). 11 $\beta$ HSD1  $-/-$  mice also have a favourable lipid and lipoprotein profile compared to controls (Morton *et al*, 2001) and a metabolically favourable adipose tissue distribution, on a C57BL6J genetic strain which is susceptible to obesity and diabetes (Morton *et al*, 2004). These findings suggest that inhibiting 11 $\beta$ HSD1 pharmacologically, and thereby lowering intracellular but not circulating glucocorticoid levels, may lead to improvements in metabolic profiles in humans.

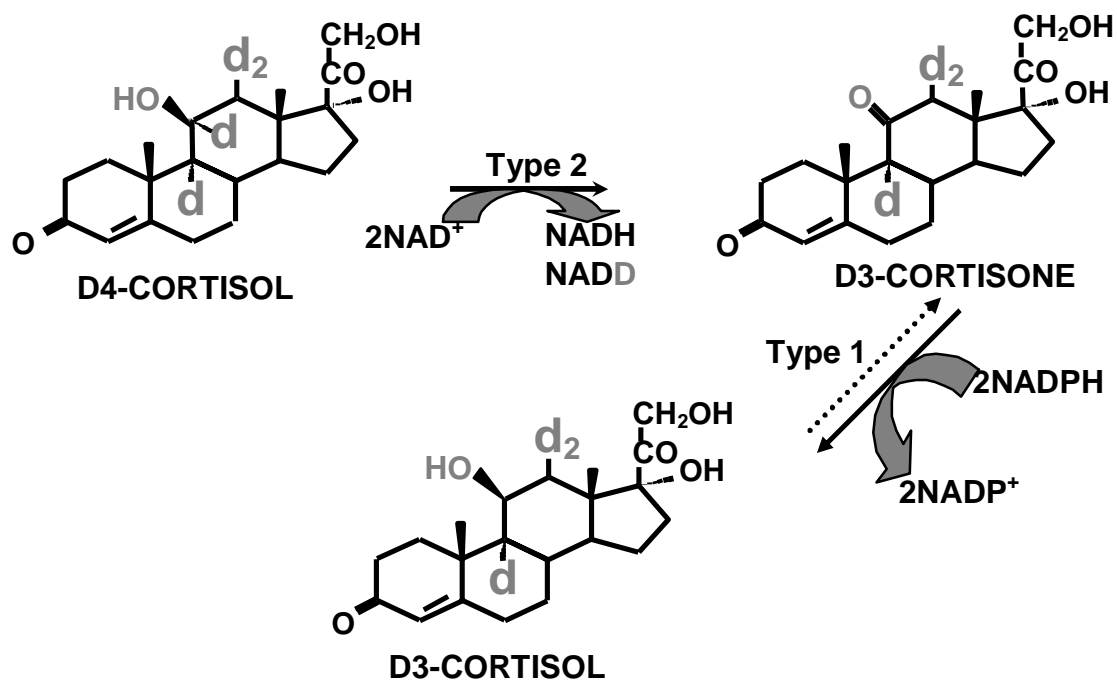
### **1.11 *In vivo* measurement of the 11 $\beta$ HSDs in humans**

Transgenic models help to predict phenotypes in humans, but given their lifelong and developmental deficiency of 11 $\beta$ HSDs and thus ‘abnormal’ tissue glucocorticoid physiology, these mice may not be a good model to directly translate into human physiology, as changes in 11 $\beta$ HSD1 expression in humans are usually as a result of specific stimuli eg obesity. Therefore, clinical studies are useful to assess 11 $\beta$ HSD1 activity *in vivo* where disruption of the enzyme from the intra-cellular environment does not occur or exposure to different

physiological stimuli from before birth, as is the case with transgenic mouse models, does not need to be accounted for.

Assessment of the 11 $\beta$ HSDs in humans is difficult. Traditionally, measurement of urinary free cortisol:cortisone ratios have been used as an index of renal 11 $\beta$ HSD2 activity (Best & Walker, 1997;Palermo *et al*, 1996), or urinary cortisol:cortisone metabolite ratios have been used to reflect predominantly intra-hepatic steroid levels. However, these ratios only reflect net balance between the activities of multiple enzymes and do not quantify the rates of turnover between cortisol and cortisone.

Stable isotope tracers allow investigators to assess metabolic pathways *in vivo* without disrupting normal physiology. One example of a stable isotope tracer is 9,11,12,12 [<sup>2</sup>H]<sub>4</sub>-cortisol (d4-cortisol), which permits measurement of cortisol generated specifically by 11 $\beta$ HSD1-reductase activity *in vivo* (Andrew *et al*, 2002). d4-Cortisol has 4 deuteriums attached in the 9, 11, 12, 12 positions of the steroid skeleton. When infused systemically, d4-cortisol is metabolised by 11 $\beta$ -dehydrogenase and loses a deuterium in the 11 $\alpha$ - position to become 9, 12, 12 [<sup>2</sup>H]<sub>3</sub>-cortisone (d3-cortisone) (Figure 1.6). d3-Cortisone is further reduced by 11 $\beta$ -reductase to form 9, 12,12 [<sup>2</sup>H]<sub>3</sub>-cortisol (d3-cortisol). Thus it is possible to distinguish between the substrates for 11 $\beta$ -dehydrogenase and the product of 11 $\beta$ -reductase and endogenous glucocorticoids. As d4-cortisol can only be metabolised to d3-cortisone but d3-cortisol cycles (d3-cortisol to d3-cortisone and vice versa), by measuring the dilution of the infused d4-cortisol by the generated d3-cortisol, the specific cortisol-generating activity of 11 $\beta$ HSD1 can be calculated (Andrew *et al*, 2002). Table 1.2 describes all published studies to date utilising this technique.



**Figure 1.6: Metabolism of the stable isotope tracer d4-cortisol by the isozymes of 11βHSD.**

When infused systemically, d4-cortisol is metabolised by 11β-dehydrogenase and loses a deuterium in the 11α- position to become 9, 12, 12 [<sup>2</sup>H]<sub>3</sub>-cortisone (d3-cortisone). d3-Cortisone is further reduced by 11β-reductase to form 9, 12,12 [<sup>2</sup>H]<sub>3</sub>-cortisol (d3-cortisol).

Several investigators have evaluated adipose tissue  $11\beta$ HSD1 cortisol regeneration rate using an infusion of d4-cortisol. Andrew *et al* used hepatic vein catheterisation to measure splanchnic cortisol production and then a single oral dose of cortisone to measure cortisol generation in the liver (Andrew *et al*, 2005). Extra-hepatic splanchnic cortisol production (including visceral adipose) was estimated to generate cortisol at a rate of 29.9nmol/min (Andrew *et al*, 2005). However, this production rate was based on modelling estimates rather than direct measurements. Two subsequent studies (Basu *et al*, 2009; Stimson *et al*, 2009) utilised arteriovenous sampling techniques across the liver and demonstrated that the liver actually accounts for all splanchnic cortisol production. Only one study to date has used a d4-cortisol infusion and arteriovenous sampling to measure cortisol production across subcutaneous adipose and this has revealed that subcutaneous adipose  $11\beta$ HSD1 generates cortisol at a rate of 15.0pmol/min/100g adipose tissue (Stimson *et al*, 2009).

Key questions regarding adipose tissue physiology in health such as glucocorticoid uptake in adipose tissue and the contribution of adipose  $11\beta$ HSD1 to the adipose glucocorticoid pool have yet to be answered using this technique and these questions will be addressed in this thesis. Furthermore,  $11\beta$ -dehydrogenase activity cannot be quantified accurately with the d4-cortisol tracer as this relies on extrapolation of d3-cortisone measurements derived from non-steady state kinetics as d3-cortisone is not infused into the plasma pool. Therefore a reliable tracer method to measure  $11\beta$ -dehydrogenase activity in the whole body and tissues is currently not available. During my PhD studies, I have developed and validated a tracer-method to measure  $11\beta$ -dehydrogenase activity *in vivo* and have subsequently used it to answer the question as to whether any  $11\beta$ HSD1-dehydrogenase activity exists in human subcutaneous adipose tissue (Chapters 4 and 5 of this thesis).

**Table 1.2 Summary of published studies (to December 2009) using the stable isotope d4-cortisol**

Reference	Subject characteristics	Duration of infusion	Interventions	T:T of infusion (d4F:F)	Whole body RaF nmol/min	Whole body Rad3F nmol/min
(Andrew <i>et al</i> , 2002)	6 healthy ♂ BMI not reported	4 hours	Placebo or CBX 1.74mg/h d4F	20:80	-	27.7 plac 21.5 CBX
(Sandeep <i>et al</i> , 2005)	6 lean ♂ BMI 24.6 6 obese ♂ BMI 36.6	5 hours	Placebo or CBX in obese group. 1.74mg/h d4F	20:80	23.1 lean 26.0 obese plac 17.2 obese CBX	13.9 lean 14.1 obese plac 9.2 obese CBX
(Wake <i>et al</i> , 2006)	9 healthy ♂ BMI 25.5	7 hours	Non-fasting. Run in period - tracers only (basal), then intervention: saline, insulin or intra-lipid. 1.74mg/h d4F	20:80	23.9 basal -12.7 saline 33.1 basal -21.5 insulin 34.2 basal -16.7 lipid	16.8 basal -16.7 saline 16.8 basal -19.3 insulin 16.7 basal -17.7 lipid
(Wake <i>et al</i> , 2007)	9 healthy ♂ BMI 27.3	5 hours	7 days either rosiglitazone, fenofibrate or placebo 1.74mg/h d4F	20:80	50.7 placebo 54.8 fenofibrate 67.0 rosiglitazone	16.3 placebo 16.1 fenofibrate 16.6 rosiglitazone



Reference	Subject characteristics	Duration of infusion	Interventions	T:T of infusion (d4F:F)	Whole body RaF nmol/min	Whole body Rad3F nmol/min
(Andrew et al, 2005)	8 healthy ♂ BMI 22.7	6.5 hours	O/N dex suppression, Dex infusion 4µg/min, Oral cortisone acetate at t=3.5h 1.74mg/h d4F	40:60	37 45 (splanchnic) 29.8 (visceral)	60 20 (splanchnic)
(Stimson et al, 2009)	6 ♂ BMI 30.8 (subcut. adipose study) 4 ♂ BMI 32.8 (visceral study)	3.5 hours (visceral study)	O/N dex suppression 1.74mg/h d4F	40:60	33.8 (subcut study) 13.5 (splanchnic)	28.3 (subcut study) 8.0 (splanchnic)

Reference	Subject characteristics	Duration of infusion	Interventions	T:T of infusion (d4F:F)	Whole body RaF nmol/min	Whole body Rad3F nmol/min
(Basu <i>et al</i> , 2004)	11 subjects. Sex n/s BMI 31	n/s	Infusion of [ <sup>3</sup> H]-glucose, somatostatin, GH and insulin. 0.19mg/h d4F	100	50 basal 66.2 with insulin	u/k
(Basu <i>et al</i> , 2005)	10 lean BMI<24 (4 ♂) 10 obese BMI>28 (4♂) 11 obese diabetics (5M:6F)	n/s	Infusion of [ <sup>3</sup> H]-glucose. 0.19mg/h d4F	100	78.2 obese diabetic 70.2 obese non-diabetic 54.7 lean	9.6 obese diabetic 10.4 obese non-diabetic 10.1 lean
(Basu <i>et al</i> , 2009)	10 obese BMI 46 (2♂)	n/s	Bariatric surgery 0.19mg/h d4F	100	Liver prod 55.2	Liver prod 5.2

Table 1.2 summarises the published studies using the d4-cortisol infusion technique. The first eight studies used an infusion of both cortisol and d4-cortisol whereas the last three studies use d4-cortisol only. The studies examine whole body cortisol / d3-cortisol production, response to interventions, and some arteriovenous sampling studies assessing tissue cortisol / d3-cortisol production. d4F= d4-cortisol; d=deuterium; F=cortisol; T:T = tracer:tracee ratio; RaF = rate of appearance of cortisol; Rad3F = rate of appearance of d3-cortisol; BMI = body mass index (kg/m<sup>2</sup>); CBX = carbenoxolone; n/s= not stated; ♂ = male; prod=production

## **1.12 Cortisol antagonism as a therapeutic target**

It has been proposed that reducing GR activation by cortisol may be of therapeutic benefit in patients with features of the metabolic syndrome. In contrast with many current therapies available for the treatment of type 2 diabetes and obesity, cortisol antagonism might be expected to be associated with improvements in all of the major features of the metabolic syndrome. Amongst many actions, lowering glucocorticoid activity would be expected to reduce hepatic glucose output (Kotelevtsev *et al*, 1997), improve lipid profiles (Morton *et al*, 2001), improve adipose insulin sensitivity and adipokine release (Masuzaki *et al*, 2001) and potentially improve insulin secretion from pancreatic  $\beta$ -cells (Davani *et al*, 2000).

However, cortisol plays vital roles in the stress response, with important effects on cellular growth and differentiation and immune modulation. A major challenge has been to devise a strategy for antagonising glucocorticoid effects on cardiometabolic parameters which does not risk inducing adrenal insufficiency at times of stress. Several approaches have been proposed.

### **1.12.1 Antagonism of adrenal steroidogenesis**

Targeting steroid biosynthesis in the adrenal is one potential method of reducing glucocorticoid activity, and has been trialled in depression (Wolkowitz *et al*, 1999). Ketoconazole, aminoglutethimide and metyrapone, traditionally used for the treatment of Cushing's syndrome, decrease plasma cortisol by inhibiting key enzymes in adrenal steroidogenesis. However, inhibiting enzymes at the level of the adrenal prevents the HPA axis adapting to stress and risks the development of a hypo-adrenal crisis during inter-current illness, and is not a safe long-term therapy.

### 1.12.2 Glucocorticoid receptor antagonism

Others groups have focused on GR antagonists such as RU38486, a glucocorticoid and progesterone receptor antagonist, which opposes the actions of glucocorticoids in peripheral tissues. Administration of RU38486 to mice with diabetes (Gettys *et al*, 1997) led to improvements in plasma glucose levels. In Cushing's syndrome, RU38486 delivered such marked improvements in glycaemic control that oral hypoglycaemic medications could be discontinued (Chu *et al*, 2001), demonstrating the beneficial metabolic effects of decreasing glucocorticoid activity.

However, RU38486 prevents GR mediated negative feedback at central sites controlling the HPA axis. Consequently, the HPA axis stimulates adrenal steroidogenesis to overcome the peripheral block imposed by this drug. Activation of the HPA axis results in the increased production of adrenal androgens and mineralocorticoids, which in turn result in hirsutism and hypertension, respectively. It is also expected that at times of physiological stress, when elevations in glucocorticoid levels are necessary, systemic GR blockade may also be problematic since it impairs GR action indiscriminately in all tissues, including cells of the immune system. Therefore, pharmacological systemic GR antagonism potentially produces undesirable systemic effects and is not a suitable long-term therapy.

To circumvent these systemic consequences, tissue-specific GR antagonists have been developed such as the Abbott liver-selective GR antagonist, which is a bile acid – RU38486 conjugate (von Geldern *et al*, 2004). The bile acid ensures that concentrations of this drug are maximised in the liver by enterohepatic circulation. Oral dosing to *ob/ob* mice led to substantial improvement in plasma glucose levels and lipid parameters (von Geldern *et al*, 2004). However, any dispersal of this compound into the systemic circulation may have

adverse effects. Indeed, this compound is no longer being developed by Abbott, perhaps this may reflect concerns about its potential abortifacient effects given that it is a progesterone - receptor antagonist, although this is purely speculative.

### **1.13 11 $\beta$ HSD1 Inhibition: pre-clinical and early clinical results**

More than 25 companies are now involved in 11 $\beta$ HSD1 inhibitor drug development programmes. In 2 years, over 70 international patent applications detailing 11 $\beta$ -HSD1 inhibitors have been published (Fotsch *et al*, 2005; Webster & Pallin, 2007).

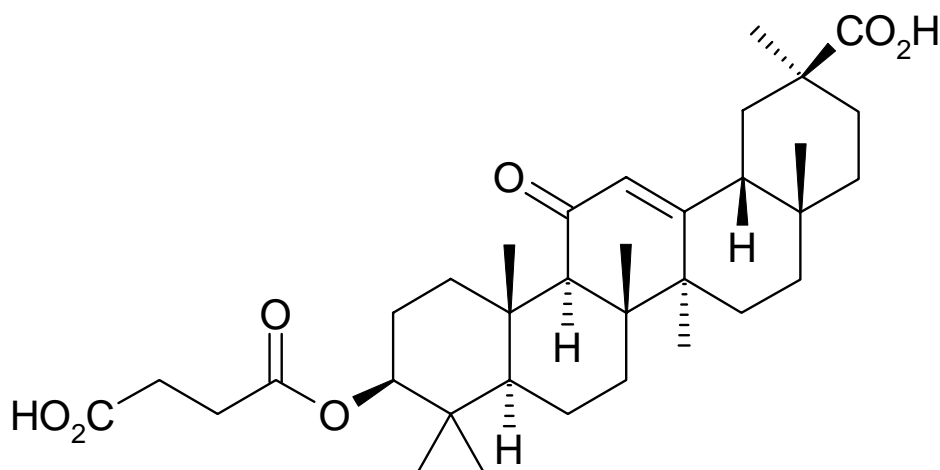
#### **1.13.1 Non-selective inhibitors: Carbenoxolone**

Carbenoxolone (CBX) is a hemisuccinate derivative of glycyrrhetic acid and a non-selective 11 $\beta$ HSD inhibitor (Figure 1.7). The effects of CBX have been assessed in obese, insulin resistant Zucker rats and in LDL-receptor knockout mice (LDLR  $-/-$ ), a model of hyperlipidaemia and obesity. In Zucker rats, CBX had no effect on fasting glucose levels or 11 $\beta$ HSD1 activity in the skeletal muscle or adipose tissue, but did inhibit 11 $\beta$ HSD1 activity in the liver (Livingstone & Walker, 2003). When administered via a subcutaneous route to LDLR  $-/-$  mice, CBX was detected in the liver and adipose tissue one hour after injection (Nuotio-Antar *et al*, 2007). These mice showed improvement in several metabolic parameters including lipid profiles, hepatic steatosis, and expression of genes involved in lipid metabolism and atherogenesis. However, improvements in fasting insulin levels were only seen in the most obese mice.

When administered to healthy lean men, CBX improved insulin sensitivity (Walker *et al*, 1995). In lean patients with type 2 diabetes, CBX administration enhanced hepatic insulin-

sensitivity, and reduced glucose production and glycogenolysis (Andrews *et al*, 2003). However, in obese human subjects, like Zucker rats (Livingstone *et al*, 2003), CBX failed to have any effect on insulin sensitivity (Sandeep *et al*, 2005), suggesting that CBX loses its beneficial effect on insulin-glucose homeostasis in the obese. Possible mechanisms for this loss of efficacy may relate to down-regulation of 11 $\beta$ HSD1 in the liver in obesity and/or the drug being unable to penetrate adipose tissue, where 11 $\beta$ HSD1 is elevated in obesity. This is in keeping with the findings of the study in LDLR<sup>-/-</sup> mice, where the authors concluded that the subcutaneous route of administration may have allowed more of the drug to bypass hepatic first-pass metabolism and thus a higher concentration of drug was in the circulation to penetrate the different tissues (the levels of CBX 1 hour post-dose were higher in liver and adipose tissue than those described previously)(Nuotio-Antar *et al*, 2007).

These studies with CBX provide important proof-of-principle evidence that 11 $\beta$ HSD1 inhibition leads to improvements in several metabolic parameters. However, CBX administration had no effects on gluconeogenesis, peripheral glucose uptake or insulin-mediated suppression of plasma free-fatty acids in individuals with type 2 diabetes (Andrews *et al*, 2003). Non-selective inhibitors, such as CBX, can also induce renal mineralocorticoid excess at higher doses. The resulting hypertension is undesirable in patients who already have type 2 diabetes, obesity and metabolic abnormalities. A therapeutic approach to circumvent CBX-induced hypertension would be to use a diuretic, such as Amiloride, in combination with CBX, as this would block the consequences of renal 11 $\beta$ HSD2 inhibition (Sandeep *et al*, 2004). However, given the potential adverse effects of blocking both 11 $\beta$ HSD isozymes, selective 11 $\beta$ HSD1 inhibitors have been developed.

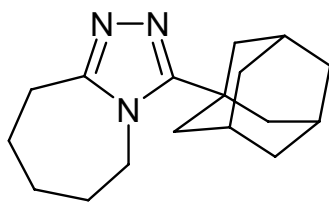


**Figure 1.7** The structure of Carbenoxolone

The non-selective 11βHSD inhibitor Carbenoxolone is orally bioavailable, able to penetrate liver and adipose (when administered subcutaneously) and improves insulin / glucose homeostasis and lipid profiles.

### 1.13.2 Selective 11 $\beta$ HSD1 inhibitors

Merck Laboratories have a potent inhibitor of 11 $\beta$ HSD1 named Compound 544 (Figure 1.8). The compound has been studied in murine models of obesity (diet-induced obesity, DIO), type 2 diabetes (high-fat streptozotocin, HF-STZ) and atherosclerosis (ApoE deletion, C57BL/6). In DIO mice, inhibition of 11 $\beta$ HSD1 led to reduction in body weight and cumulative food intake (Hermanowski-Vosatka *et al*, 2005) with preferential loss of central fat mass. However, there was little effect on body weight in HF/STZ model (Hermanowski-Vosatka *et al*, 2005). With regards to type 2 diabetes, in DIO mice Compound 544 lowered fasting glucose and insulin levels below that seen in lean controls (Hermanowski-Vosatka *et al*, 2005), and lowered fasting and post-prandial glucose excursions and improved insulin sensitivity in HF/STZ mice. In atherosclerosis, encouraging improvements were seen in lipid profiles. Triglycerides were lowered in all mouse models studied, and fatty acids and cholesterol improved in some. Inhibition of 11 $\beta$ HSD1 also slowed atherosclerotic plaque progression in ApoE  $-/-$  mice fed on a high-fat atherogenic diet (Hermanowski-Vosatka *et al*, 2005). There are no reported clinical studies involving Compound 544.





Thiazoles from Biovitrum are species-selective inhibitors of 11 $\beta$ HSD1. In pre-clinical studies, a single oral bolus dose of BVT 2733 resulted in inhibition of hepatic 11 $\beta$ HSD1 activity in hyperglycaemic KKA<sup>y</sup> mice (Alberts *et al*, 2002). Subcutaneous drug-delivery resulted in significant lowering of glucose and insulin levels and lowering of hepatic mRNA of G6Pase and PEPCK, reducing hepatic glucose production (Alberts *et al*, 2002).

An Amgen – Biovitrum compound, BVT 3498 reached clinical development. A phase one trial in healthy volunteers was undertaken in 2002 and a phase 2a double-blind, placebo-controlled trial in individuals with type 2 diabetes in 2003. However, in 2004, Amgen announced that their series of Amgen-Biovitrum compounds had failed in phase 2 trials.

Incyte is a further company that has progressed from pre-clinical to clinical trials with their orally administered 11 $\beta$ HSD1 inhibitors. A phase 1 double-blinded placebo-controlled clinical study of INCB13739 to evaluate pharmacokinetics and the ability of the drug to inhibit 11 $\beta$ HSD1 activity in adipose tissue and liver of subjects with a high BMI and insulin resistance has been completed. A phase 2a study using a 2-phase insulin clamp in 24 volunteers was run in 2007 and demonstrated clinical improvements in glucose and lipid profiles in people with diabetes. A further phase 2b efficacy trial in 2008 demonstrated significant improvements in glucose control, insulin sensitivity and total cholesterol in patients with type 2 diabetes.

### **1.13.3 Other compounds**

Other compounds in medical and everyday use have also proven to be useful inhibitors. The bile acid chenodeoxycholic acid (Diederich *et al*, 2000), coffee (Atanasov *et al*, 2006) and flavanone and its derivatives (found in orange and yellow fruit) can inhibit 11 $\beta$ HSD1

(Schweizer *et al*, 2003). As yet, there are no pre-clinical or clinical trials in the scientific literature describing these compounds as 11 $\beta$ HSD1 inhibitors.

### **1.14 Effects of 11 $\beta$ HSD1 inhibition beyond Metabolic Syndrome**

In addition to its expression in liver and adipose tissue, 11 $\beta$ HSD1 is active in a number of other cells and tissues (Tomlinson *et al*, 2004). Accumulating evidence from 11 $\beta$ HSD1  $-/-$  mice and from pharmacological inhibition of 11 $\beta$ HSD1 suggests that the enzyme is important in modulating effects of glucocorticoids not only on metabolism, but also on immunomodulatory, cognitive and other tissue functions. Small molecule selective 11 $\beta$ HSD1 inhibitors are likely to affect these pathways. This may produce beneficial effects by reducing glucocorticoid action in skeletal muscle (Jang *et al*, 2007), the eye and brain. For example, carbenoxolone (CBX) improves cognition in elderly men, including those with type 2 diabetes (Sandeep *et al*, 2004), an effect which is mimicked by Merck's 11 $\beta$ HSD1 inhibitors in mice. CBX may also improve intra-ocular hypertension in people with glaucoma (Rauz *et al*, 2003). However, the effects of inhibitors on other tissues such as macrophages and vascular tissue, and indeed other sites in the brain, may or may not be beneficial.

Since 11 $\beta$ HSD1 is expressed in macrophages (Thieringer *et al*, 2001), and glucocorticoids promote macrophage consumption of apoptotic neutrophils and hence promote resolution of inflammation, one potential side effect of 11 $\beta$ HSD1 inhibition is to exaggerate innate immune responses. 11 $\beta$ HSD1  $-/-$  mice show delayed macrophage-phagocytic ability (Gilmour *et al*, 2006) and delayed resolution of inflammation in experimental arthritis (Chapman *et al*, 2006). Thus 11 $\beta$ HSD1 inhibitors may impair the inflammatory response and

in individuals susceptible to soft tissue infections, such as those with type 2 diabetes, this might lead to delayed wound healing.

A further site of 11 $\beta$ HSD1 expression is the blood vessel wall. Supra-physiological doses of glucocorticoids are known to inhibit angiogenesis, as illustrated by the poor wound healing seen in Cushing's syndrome. 11 $\beta$ HSD1  $-/-$  mice show enhanced angiogenesis in wounds and in infarcted myocardium (Small *et al*, 2005). 11 $\beta$ HSD1 inhibitors might, therefore, promote angiogenesis. However, the mechanism for this effect and its relevance in different circumstances of angiogenesis is unknown. It could be potentially beneficial in wound healing and infarcted areas of tissue, but potentially disadvantageous in promoting cancer growth, and diabetic retinopathy. The latter is a particular concern because people with diabetes are most likely to gain from the beneficial metabolic effects of 11 $\beta$ HSD1 inhibitors.

In the brain, 11 $\beta$ HSD1 is expressed in key areas including the hippocampus (Sandeep *et al*, 2004) and most small molecule 11 $\beta$ HSD1 inhibitors penetrate this tissue. There have been concerns that inhibition of hippocampal 11 $\beta$ HSD1 will reduce local tissue glucocorticoid levels and reduce the negative feedback suppression of the HPA (Walker & Andrew, 2006). The expected effect of this is a compensatory increase in cortisol levels, and indeed mild elevations in basal corticosterone levels were found in 11 $\beta$ HSD1  $-/-$  mice (Kotelevtsev *et al*, 1997). However, despite the elevated corticosterone, these mice retain their protected metabolic profile and thus this may not be a clinically relevant problem for small molecule 11 $\beta$ HSD1 inhibitors. Moreover, corticosterone concentrations were normal in 11 $\beta$ HSD1  $-/-$  mice crossed onto a C57BL6 genetic background (Paterson *et al*, 2007).

11 $\beta$ HSD1 may be bidirectional in some metabolically important 11 $\beta$ HSD1-inhibitor target-tissues eg adipose tissue (Wake *et al*, 2006). If 11 $\beta$ HSD1-inhibitors are not 11 $\beta$ HSD1-

reductase selective, any 11 $\beta$ HSD1-dehydrogenase activity in these tissues may be inhibited which may actually increase tissue glucocorticoid levels. It is not clear if most of the published inhibitor-compounds are indeed reductase-selective. Therefore discrepancies in published clinical efficacy might be explained by non-selective compounds inhibiting 11 $\beta$ HSD1-dehydrogenase activity in adipose tissue (or liver).

## 1.15 Conclusion

Transgenic mouse models have demonstrated that dysregulation of glucocorticoid production in tissues by 11 $\beta$ HSD1 leads to obesity and metabolic syndrome. In human idiopathic obesity, circulating cortisol levels are not elevated but glucocorticoid production by 11 $\beta$ HSD1 in tissues is altered. Pre-clinical and clinical studies of 11 $\beta$ HSD1-inhibitors have shown promising metabolic results. However several key questions regarding adipose glucocorticoid regulation in health remain unanswered to ensure that these drugs provide metabolic benefits in humans without causing major side effects. These unanswered questions in human adipose tissue are the focus of the experiments in this thesis.

## 1.16 Hypothesis

The hypotheses examined in this PhD were as follows:

1. Absolute levels of glucocorticoids in adipose tissue and the contribution of 11 $\beta$ HSD1 to the intra-adipose glucocorticoid pool can be accurately quantified using the stable isotope tracer d4-cortisol.
2. 11 $\beta$ HSD1-dehydrogenase activity can be measured *in vivo* using stable isotope tracers.

3. Adipose tissue possess some potentially metabolically protective 11 $\beta$ HSD1-dehydrogenase activity leading to cortisol inactivation in adipose tissue, which can be quantified using stable isotope tracers and modulated through inducing hyperinsulinaemia.

## 1.17 Aims

1. To develop a method to measure adipose tissue glucocorticoids using liquid chromatography tandem mass spectrometry (LC-MS/MS) (discussed in Chapter 3).
2. To assess glucocorticoid uptake in adipose tissue using the stable isotope tracer 9,11,12,12 [ $^2\text{H}$ ]<sub>4</sub>-cortisol (discussed in Chapter 3).
3. To determine the relative contribution of the HPA axis and adipose tissue 11 $\beta$ HSD1 to the adipose glucocorticoid pool (discussed in Chapter 3).
4. To develop a stable isotope tracer method with 1,2 [ $^2\text{H}$ ]<sub>2</sub>-cortisone to measure 11 $\beta$ -dehydrogenase activity *in vivo* (discussed in Chapter 4).
5. To measure 11 $\beta$ HSD1 directionality in adipose tissue and skeletal muscle *in vivo* using the stable isotope tracers 9,11,12,12 [ $^2\text{H}$ ]<sub>4</sub>-cortisol and 1,2 [ $^2\text{H}$ ]<sub>2</sub>-cortisone (discussed in Chapter 5).
6. To assess the effect of increased glucose flux into adipose tissue and skeletal muscle on 11 $\beta$ HSD1 activity and directionality (discussed in Chapter 5).

## **Chapter 2**

### **Materials and Methods**

## 2.1 Introduction to materials and methods

All clinical measurements, sample collection, assays and analysis were carried out by the author after appropriate advice, except those detailed in the declaration page iv. Methods already in routine use within this research unit were modified and optimised for tracer studies in human tissues and plasma and human bioassays. *De novo* methods developed include measurement of tissue (adipose and liver) and salivary glucocorticoids using liquid chromatography tandem mass spectrometry (LC-MS/MS). Where room temperature is stated this corresponds to a temperature of 18-22°C.

## 2.2 Equipment

### a) Centrifuges:

- D2-cortisone studies (hepatic cytosol preparation), Chapter 2: Beckman Optima TLX Ultracentrifuge, (Beckman Coulter, High Wycombe, UK).
- Method development, adipose glucocorticoid extraction, Chapter 3: Eppendorf centrifuge 5415C (Cambridge, UK).
- Tissue tracer study, Chapter 3 (plasma and adipose): Eppendorf centrifuge 5810R, (Cambridge, UK).
- D2-cortisone studies (plasma, saliva), Chapter 4: Sigma 4K15 centrifuge, (SciQuip, Shrewsbury, UK).
- Oxford arterio-venous sampling study (plasma), Chapter 5: Eppendorf 5702R centrifuge (Cambridge, UK).

b) Beta-scintillation counter:

- Tri-carb 2100TR liquid scintillation analyser, (Packard Instruments, Chicago, IL).

c) pH meter:

- pH 210 microprocessor pH meter, (Hanna Instruments, Leighton Buzzard, UK).

d) Homogeniser:

- Pro200 homogeniser (Pro scientific Inc, Oxford, CT).

e) Vortex:

- MS2 Minishaker (IKA, Staufen, Germany).

f) Sonicator:

- Branson sonicator model 1210 (Branson, Danbury, CT).

g) Whole blood oxygen saturation:

- GEM OPL instrument (Instrumentation laboratory, Bedford, MA).

h) Haematocrit:

- Haematospin 1300 instrument (Hawksley, Lancing, Sussex, UK).



## 2.3 Sources of materials

All reagents were obtained from Sigma (Poole, UK) or VWR (Lutterworth, UK) unless otherwise specified. All solvents were glass distilled HPLC grade from Fisher Scientific (Loughborough, UK).

### 2.3.1 Commonly used buffers

a) Sodium phosphate buffer: 1 part sodium dihydrogenphosphate ( $\text{NaH}_2\text{PO}_4$ ) (0.2M), 3 parts disodiumhydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ) (0.6M), 5mM ethylenediaminetetraacetic acid (EDTA) dissolved in distilled water and adjusted to pH 7.0 using concentrated orthophosphoric acid.

b) Sucrose / HEPES buffer: 250mM sucrose, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), dissolved in distilled water, and adjusted to pH 7.5 using sodium hydroxide (1M), and stored at 4°C.

c) 40mM Sodium phosphate buffer: 40mM disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ), dissolved in distilled water, and adjusted to pH 7.4 using concentrated orthophosphoric acid, and stored at 4°C.

d) Sucrose / sodium phosphate buffer: 320nM sucrose, 1mM dithiothreitol (DTT) dissolved in sodium phosphate buffer (40mM, solution c), made fresh for each assay.

e) Potassium phosphate buffer: 50mM potassium dihydrogenphosphate ( $\text{KH}_2\text{PO}_4$ ), 1mM EDTA dissolved in distilled water (adjusted to pH 7.4 using potassium hydroxide (1M), stored at 4°C).

f) Sodium acetate buffer: 0.2M sodium acetate dissolved in distilled water (adjusted to pH 4.6 using glacial acetic acid, and stored at 4°C).

### **2.3.2 Drugs for clinical studies**

#### ***Stable isotopically labelled tracers***

All stable isotopically labelled tracers were obtained from Cambridge Isotope Laboratories (CIL, Andover, MA.). Chemical and isotopic purity were assessed by the manufacturer.

a) 1,2- $[\text{}^2\text{H}]_2$ -cortisone powder (d2-cortisone): >96.3% purity and 98% isotopic enrichment as determined by single spot thin layer chromatography (TLC).

b) 1,2- $[\text{}^2\text{H}]_2$ -cortisol powder (d2-cortisol): >99% purity and 98.9% isotopic enrichment as determined by  $^1\text{H}$  nuclear magnetic resonance.

c) 9,11,12,12- $[\text{}^2\text{H}]_4$ -cortisol powder (d4-cortisol): >98% purity and 98.6% isotopic enrichment as determined by gas chromatography mass spectrometry.

### ***Unlabelled hydrocortisone***

Hydrocortisone (chromatographic standard) powder: 100% purity by TLC, (Calbiochem, Nottingham, UK).

### ***Diluents***

a) Saline (NaCl): sodium chloride (0.9% w/v) (Baxter, Newbury, UK), containing 77mmol/500ml (154mM) sodium and 77mmol/500ml (154mM) chloride.

b) Dehydrated alcohol BP: Ethanol (100% v/v) (Martindale, Brentwood, UK).

### ***Insulin / glucose***

a) Dextrose: dextrose (20% w/v) (Baxter, Newbury, UK), containing 200g anhydrous dextrose / 1000ml (1.1M).

b) Actrapid insulin: Actrapid (NovoNordisk, Crawley, UK), containing 1000units / ml.

### **2.3.3 Drugs and reagents for *in vitro* assays**

a) Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH): Required in a solution with the final concentration in the assay of 2mM. Therefore prepared initially in a solution with a concentration of 10mM and dissolved in potassium phosphate buffer (1ml).

b) D-Glucose-6-phosphate dipotassium salt hydrate: Required in a solution with the final concentration in the assay of 5mM. Therefore prepared initially in a solution with a concentration of 25mM and dissolved in potassium phosphate buffer (1ml). Solutions a) and b) were prepared fresh for each assay and stored at 4°C for no longer than 2 hours and protected from the light.

c) 1,2n-[<sup>3</sup>H]<sub>2</sub>-Cortisone: 1,2n-[<sup>3</sup>H]<sub>2</sub>-cortisone was obtained from Amersham Radio chemicals (GE Healthcare UK Ltd, Little Chalfont, UK). Commercial stock solutions of 1, 2n-[<sup>3</sup>H]<sub>2</sub>-cortisone were obtained in ethanol (with concentrations of 19.6 pmol/ml and specific activity of 0.037MBq/μl) and stored at -20°C.

d) 1, 2, 6, 7-[<sup>3</sup>H]<sub>4</sub>-Corticosterone: 1, 2, 6, 7-[<sup>3</sup>H]<sub>4</sub>-corticosterone was also obtained from Amersham Radio chemicals. Commercial stock solutions of 1, 2, 6, 7-[<sup>3</sup>H]<sub>4</sub>-corticosterone were obtained in a toluene: ethanol (9:1) solution (with concentrations of 13.7 pmol/ml and specific activity of 0.037MBq/μl) and stored at -20°C.

e) Unlabelled steroids: Corticosterone, cortisol, cortisone were obtained from Sigma (Poole, UK) and epi-cortisol, epi-tetrahydrocortisol, epi-corticosterone, epi-tetrahydrocorticosterone, 3α,5β-tetrahydrocortisol, 3α,5β-tetrahydrocortisone, 5β-dihydrocortisone, 20α- and 20β-dihydrocortisone and α- and β-cortolone were obtained from Steraloids (Newport, RI). Each of these steroids were weighed and dissolved in methanol (final concentration 1mg/ml) and stored at -20°C.

### **2.3.4 Drugs and reagents for tissue culture**

a) Dulbecco's Modified Eagle's Medium (DMEM): Containing fetal calf serum (FCS) (10%) (stripped of steroids with charcoal), glucose 4.5g/L, glutamine (2mM) and penicillin (1% w/v)/ streptomycin (1% w/v), obtained from Lonza (Slough, UK).

b) Trypsin: Trypsin (200mg/l) -EDTA, (Lonza, Slough, UK).

c) Charcoal: Charcoal, dextran coated (Sigma, Poole, UK).

#### ***Charcoal stripped serum***

To prepare serum stripped of steroids, fetal calf serum (500ml) and dextran coated charcoal (5g) were mixed (~16 hours) at 4°C. The solution was filtered through a 250µm filter funnel, separated into aliquots and stored at -20°C until use.

### **2.3.5 Animal tissues**

Animals were obtained from Harlan Laboratories (Bicester, UK) and maintained under standard conditions, 12 hour dark / light cycle, constant temperature / humidity, with free access to normal chow in the Biomedical Research Facility, Little France. Maintenance conformed to good practice as dictated by the UK Home Office.

## 2.4 In vitro bioactivity assays

### 2.4.1 5 $\beta$ -reductase assay

#### *Tissue homogenisation*

Liver (100mg) was cut into small pieces on a bed of dry ice, weighed, transferred into a 1.5ml tube and sucrose / HEPES buffer (1ml) added. Liver pieces were stored on wet ice prior to mechanical homogenisation (3 x 10 second bursts placing on wet ice in between). When using human tissues, homogenisation was carried out in a Class 1 bio-safety cabinet. Aliquots (250 $\mu$ l) of rodent hepatic homogenate were diluted to a specific protein content with sucrose / HEPES homogenisation buffer and stored at -80°C. Human hepatic homogenates were stored undiluted at -80°C in 250 $\mu$ l aliquots.

#### *Cytosol preparation*

Human and rodent hepatic homogenates were subject to centrifugation (94 x g, 10min, 4°C), the tissue pellet discarded and supernatant transferred into ultra-centrifuge tubes. The supernatant underwent further centrifugation (34 000 x g, 30min, and 124 000 x g, 60min, 4°C). Cytosol (the final supernatant) was stored on wet ice prior to measuring the protein concentration.

### ***Determination of protein concentrations***

The protein concentrations of hepatic homogenates were determined using a colorimetric Bio-Rad DC protein assay kit (Bio-Rad, Hemel Hempstead, UK). Solutions of standards (0.1–1.4 mg/ml) were prepared in triplicate in distilled water from a stock protein solution (2mg/ml bovine serum albumin (BSA- fraction V) in water). A solution of sodium dodecyl sulfate (SDS) (1%) in water was used as a blank in the assay. 5µl of either SDS (1%) or protein standards (in triplicate) were added to the 96-well plate. Unknown protein solutions were also added in triplicate. Protein assay Reagent A (3ml) was mixed with Reagent S (60µl) to form working Reagent A. Working Reagent A (25µl) was added into each of the wells. Dye Reagent B was subsequently added (200µl). The plate was allowed to stand at room temperature (15 min) and absorbance of samples at  $\lambda$ 690nm was measured using a spectrophotometer. A standard curve was plotted with absorbance on the y axis vs concentration on the x axis and a line of best fit drawn. The concentration of protein in each sample was calculated from the equation of the regression line. Data were deemed acceptable if the regression was >0.99 and % coefficient of variation between triplicates was <10%.

### ***Assay development with rat hepatic cytosol***

The assay was adapted from one reported to quantify 5 $\beta$ -reductase activity in rat (Okuda & Okuda, 1984) and human liver (Iyer *et al*, 1990). Practice assays were performed with rat hepatic cytosol to determine the optimum amount of protein to use in the assay, the best buffer to use, incubation time, and optimise the chromatographic conditions.

## ***Metabolism of steroids by 5 $\beta$ -reductase in human hepatic cytosol***

Cytosols were incubated in duplicate (37°C, 16h) in potassium phosphate buffer with substrate (10 $\mu$ M) and [ $^3$ H]-substrate (10nM) in the presence of a co-factor generating system to assess the metabolism of the substrate by 5 $\beta$ -reductase and 3 $\alpha$ HSD. The co-factor generating system was prepared by adding NADPH (2mM), glucose-6-phosphate (5mM) and glucose-6-phosphate dehydrogenase (0.5 unit/250 $\mu$ l) to the cytosol extract and reaction substrate. The reaction was terminated by mixing with ethyl acetate (10 vol) and steroids extracted as per section 2.6.1. Dried organic extracts were dissolved in mobile phase (200 $\mu$ l) and analysed by HPLC with radio-scintillation detection as per section 2.8.1. Experiments without co-factor or cytosol served as controls. Products formed from substrate were identified by comparison of their retention times with those of unlabelled standards.

## **2.5 Cell culture**

### **2.5.1. Source of cells**

Human embryonic kidney cells (HEK293) stably transfected with human 11 $\beta$ HSD1 (HEK293h11 $\beta$ HSD1) were obtained from Dr Scott Webster, Drug Discovery Manager, University of Edinburgh (Webster *et al*, 2007).

### **2.5.2 HEK 293 cell culture**

HEK293h11 $\beta$ HSD1 cells were maintained in poly-D-lysine pre-treated flasks in standard tissue culture conditions (37°C, 95% O<sub>2</sub>: 5% CO<sub>2</sub>), in DMEM (15ml) containing glutamine (2mM) and penicillin (1% w/v)/ streptomycin (1% w/v) and fetal calf serum (FCS) (10% v/v).



Cells were passaged once they reached confluence (approximately twice weekly). Cells at their 3rd-4th passage were used for assays unless otherwise stated.

To passage the cells, DMEM was aspirated off and the cells washed with phosphate-buffered saline (5ml). Trypsin / EDTA (3ml) was added and the cells incubated (37°C) for 2-3 minutes. The cells were dislodged and observed under the microscope. Trypsin was neutralised with an equal volume of medium, and the cells then subject to centrifugation (1000 x g, 5 mins, room temp, 50ml screw-cap conical Falcon tube (BD Biosciences, Oxford, UK)) to remove any debris. The media was removed and the cell pellet re-suspended fresh media. The required volume of suspension was transferred into each flask and the flask topped-up to 15ml of media in total.

To seed 60mm plates, cells were trypsinised as per passaging protocol, and the trypsin neutralised with an equal volume of medium. Medium containing cells (200µl) was removed and placed on a haemocytometer under a light microscope for counting. For each experiment  $2 \times 10^5$  cells were seeded in each well.

### **2.5.3 Experimental conditions**

#### ***In vitro metabolism of steroids by 11 $\beta$ -reductase***

HEK293h11 $\beta$ HSD1 cells ( $2 \times 10^5$ ) were incubated (37°C, 5%CO<sub>2</sub>) in duplicate in DMEM (4ml) containing stripped fetal calf serum (10% v/v), glutamine (2mM), penicillin (1% w/v)/streptomycin (1% w/v) and substrate (2µM). Medium (250µl) was harvested (1-24h) and steroids extracted as per section 2.6.2. Steroids were dissolved in mobile phase (100µl), and analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) as per section 2.8.2. Controls were included without cells or addition of steroid. A standard curve

was prepared to quantify steroids extracted from medium, representing a concentration range of 0-400ng, with 500ng of internal standard (epi-cortisol).

### ***Assessment for a primary isotope effect on 11 $\beta$ -reductase***

The presence of a stable isotope label on a substrate may cause an alteration in reaction kinetics. To further test for a 'primary isotope' effect using a competition approach, HEK293/h11 $\beta$ HSD1 cells ( $2 \times 10^5$ ) were incubated (6-24h) in duplicate with [ $^3$ H]-substrate (5nM) and increasing concentrations of deuterated or non-deuterated substrate (0nM - 4955nM). Medium was extracted as per section 2.6.2 using conditioned Sep-Pak C18 columns and steroids dissolved in mobile phase (1ml) in preparation for HPLC analysis as per section 2.8.1. Controls were included without cells or addition of steroid.

## **2.6 Extraction of glucocorticoids from experimental matrices**

### **2.6.1 Bioactivity assay buffers**

Cytosol was mixed with ethyl acetate (10 vol), the organic layer removed and dried under oxygen-free nitrogen (OFN, 60°C). Organic extracts were dissolved in mobile phase (200 $\mu$ l) and analysed by HPLC as per section 2.8.1.

### **2.6.2 Cell culture medium**

Samples of cell culture medium (250 $\mu$ l) were thawed at room temperature (30 min) prior to extraction and enriched with internal standard (500ng). Steroids were extracted using conditioned Sep-Pak C18 columns (300mg, Waters Millipore, Watford, UK). To prepare

columns for use, methanol (5ml) was added to wash the column followed by water (5ml) to separate the C18 chains. The medium was applied, allowed to elute and then the column was washed once with water (5ml) and steroids eluted using methanol (2ml). The eluate was dried, reconstituted in water (200µl) and steroids extracted in ethyl acetate (2ml). The organic layer was dried again and dissolved in mobile phase (100µl or 1ml) for analysis by LC-MS/MS or HPLC respectively (sections 2.8.2 or 2.8.1).

### **2.6.3 Plasma**

#### ***Source***

Plasma was obtained during the clinical studies detailed in Chapters 3-5 after written informed consent was obtained.

#### ***Experimental***

To quantify deuterated tracers and endogenous glucocorticoids from plasma stock solutions of cortisone, cortisol, epi-cortisol, d2-cortisone, d2-cortisol and d4-cortisol in methanol were prepared. From these stock solutions, further solutions containing mixtures of the steroids were prepared for standard and enrichment curves for the analysis.

The standard curve to quantify cortisol and cortisone represented a range of amounts from 0-400ng with 500ng of internal standard (epi-cortisol). The enrichment curve represented a range from 0-20%. Standard curves to quantify d4-cortisol and d2-cortisone represented a range of amounts from 0-200ng. Enrichment curves for d2-cortisone ranged from 0-20%. Table 2.1 shows an example of a standard and enrichment curve for d4-cortisol and cortisol.

Steroid solutions were placed in glass tubes, reduced to dryness under a stream of OFN (60°C), dissolved in methanol (50µl) and distilled water (200µl) was added to mimic plasma and retain any hydrophilic molecules. Chloroform (2ml) was added, mixed and the water removed using a glass pipette and discarded. The remaining steroidal extract in chloroform was transferred into glass vials and reduced to dryness (60°C, OFN). The extract was dissolved in a mobile phase (50µl) in preparation for analysis by LC-MS/MS (section 2.8.2).

Plasma samples were thawed at room temperature (30 min) prior to extraction. In glass tubes, plasma (1.5-2.0ml) was enriched with epi-cortisol (500ng) and chloroform (15-20ml) added and mixed thoroughly. Excess plasma and lipid were removed using a glass pipette and discarded. The chloroform layer containing steroids was decanted into a clean glass tube, and reduced to dryness (OFN, 60°C). Dried steroidal extracts were dissolved in mobile phase (50µl) and placed in auto sampler vials in preparation for analysis by LC-MS/MS (section 2.8.2).

**Table 2.1 Example of standard and enrichment curves used to quantify steroids in plasma and tissues**

In the standard curve a fixed quantity of internal standard (epi-cortisol) is added to increasing amounts of the analyte (eg cortisol and d4-cortisol). The enrichment curve reflects ratio of the tracer (eg d4-cortisol) to the tracee (eg cortisol).

STANDARD CURVE			ENRICHMENT CURVE		
Cortisol (ng)	Epi-cortisol (ng)	D4-cortisol (ng)	Enrichment (%)	Cortisol (ng)	D4-cortisol (ng)
0	500	0	0	500	0
10	500	5	2.4	500	12.5
20	500	10	4.8	500	25
30	500	15	9.1	500	50
50	500	25	13.0	500	75
100	500	50	16.7	500	100
200	500	100	20	500	125
300	500	150			
400	500	200			

#### **2.6.4 Saliva**

##### ***Source***

Saliva was obtained during the clinical study detailed in Chapter 4 after written informed consent was obtained.

##### ***Experimental***

Frozen saliva samples (1ml) were thawed at room temperature, subject to centrifugation (3200 x g, 4°C, 3 min.) and enriched with 500ng epi-cortisol (internal standard). Salivary glucocorticoids were extracted into chloroform (10ml) as per plasma (section 2.6.3) and analysed using LC-MS/MS (section 2.8.2).

#### **2.6.5 Urine**

##### ***Source***

Urine was obtained during the clinical study detailed in Chapter 4 after written informed consent was obtained.

##### ***Experimental***

Steroids in urine (15ml) or water (containing known amounts of steroids) both containing internal standard (5µg epi-cortisol and 30µg epi-tetrahydrocortisol) were retained on conditioned Sep-Pak C18 columns (300mg) (section 2.6.2) and steroids subsequently eluted in methanol (2ml). Glucocorticoid conjugates were hydrolysed in sodium acetate buffer,

(2ml) using  $\beta$ -glucuronidase (Helix pomatia, Type H-2, 85,000 units/mL; 100 $\mu$ l; 37°C for 48 hours). Hydrolysates were passed through conditioned Sep-Pak C18 columns to retain steroids and a methanolic eluate reduced to dryness under OFN (60°C). The residue was re-constituted in water (200 $\mu$ l), re-extracted with ethyl acetate (2ml) and the organic layer dried (OFN, 60°C). The steroids obtained were derivatised to form methoxime-trimethylsilyl (MO-TMS) derivatives (section 2.7.2) (Best & Walker, 1997) and analysed using GC-MS (section 2.8.4). The standard curves to quantify cortisol and cortisone represented a range of amounts from 0-30 $\mu$ g. The standard curves to quantify  $\alpha$ - and  $\beta$ -tetrahydrocortisol and tetrahydrocortisone represented a range of amounts from 0-300 $\mu$ g.

## **2.6.6 Adipose**

### ***Experimental conditions***

A protocol to extract glucocorticoids from adipose tissue was developed and optimised (further details given in Chapter 3). The final method used was as follows:

To release intracellular glucocorticoids, adipose samples (250mg) were mechanically homogenised in ethyl acetate (1ml), enriched with internal standard (epi-cortisol 250ng), and the homogenate dripped onto chilled ethanol: glacial acetic acid: water (95:3:2 v/v) (10ml) and stored at -80°C overnight. Samples attained 4°C, before being sonicated (8 x 15 sec bursts) and debris separated by centrifugation (3000x g, 30 min, 4°C). The supernatant was reduced to dryness (OFN, 60°C), methanol (10ml) added and stored at -80°C overnight. To remove oils, tissue extracts attained room temperature and then hexane (10mls) was added and mixed. The hexane layer was discarded and the remaining methanol removed by evaporation. Water (400 $\mu$ l) and ethyl acetate (4ml) were added to the dried steroidal extract, the organic layer removed, dried and the residue re-suspended in 30% methanol (5ml). Adipose steroidal extracts (5ml) were applied to conditioned C18 Bond Elut columns

(Varian, Oxford, UK, 2g) (as described below), eluted in methanol (5ml), dried (OFN, 60°C) and dissolved in a mobile phase (50µl) in preparation for analysis by LC-MS/MS (section 2.8.2).

To prepare a C18 Bond Elut cartridges (2g, Varian, Oxford, UK) the following method was used. Methanol (2 x 10ml) and water (2 x 10ml) were applied to the column under a small amount of vacuum (3-4 iu Hg). The adipose extracts were applied to the column and the steroids eluted in methanol (5ml) after a further wash with water (2 x 10ml).

### **2.6.7 Infusates**

To extract steroids from infusates a standard curve was prepared as per plasma extractions. Prior to starting the extraction, the expected amount of glucocorticoid in the infusate was calculated to ensure the values were within the range of the standard curve (5µl 60:40 cortisol: d4-cortisol infusate = 120ng of cortisol mixture (72ng cortisol and 48ng d4-cortisol)). For the samples, infusate (5µl) and water (195µl) were added to glass tubes and enriched with epi-cortisol (500ng). After mixing, chloroform (2ml) was added and the contents of the glass tubes mixed further. The layers were allowed to separate, and the water removed from the extract using a glass pipette. The remaining sample was transferred to a glass vial, reduced to dryness (OFN, 60°C) and dissolved in mobile phase (50µl) in preparation for LC-MS/MS (section 2.8.2).



## **2.7 Derivatisation of glucocorticoids for analysis by gas chromatography mass spectrometry**

### **2.7.1 Derivatisation agents**

a) Lipidex 5000 (Packard, Pangbourne, UK): Lipidex 5000 suspended in methanol, was filtered, the methanol discarded and the beads re-suspended in mobile phase (b) (200ml) below and stored in the dark at room temperature.

b) Lipidex mobile phase: 98% cyclohexane (v/v), 1% hexamethyldisilazane (v/v), 1% pyridine (v/v).

c) Methoxyamine in pyridine: methoxyamine hydrochloride in pyridine (2% w/v).

d) Trimethylsilylimidazole (TMSI): undiluted.

### **2.7.2 Preparation of Lipidex 5000 size occlusion columns**

To prepare a Lipidex 5000 column, a glass Pasteur pipette was plugged with a small amount of silanised glass wool. Lipidex 5000 (1ml) in mobile phase was added to the column and excess mobile phase allowed to elute. The trapped matrix was subjected to three washes with mobile phase (3 x 1ml).

### 2.7.3 Derivatisation conditions

The following method was used to form methoxime-trimethylsilyl (MO-TMS) derivatives (Best & Walker, 1997): methoxyamine hydrochloride in pyridine (50µl 2% w/v) was added to the dried steroidal extract, mixed and heated in an oven (60°C, 30 min). The extract was reduced to dryness; trimethylsilylimidazole (50µl) added, mixed and heated (100°C, 2 hours). The sample was re-suspended in a mobile phase of cyclohexane: hexamethyldisilazane: pyridine (98:1:1) (1ml) before being passed through a Lipidex 5000 column. The glass Reacti-vial was washed with further mobile phase (2 x 0.5ml), which was also applied to the column. The eluate was collected, reduced to dryness (OFN, 60°C) and re-suspended in decane (50µl).

## 2.8 Chromatographic analysis

### 2.8.1 High Performance Liquid Chromatography (HPLC) with radio-detection

#### *Instrument*

Analysis of tritiated steroids was achieved using a Waters HPLC 600S pump (Elstree, UK), 717 plus Autosampler and a β-scintillation counter (Berthold LB509 detector (Harpenden, UK). When simultaneous analysis of non-tritiated steroids was required a Waters 2487 UV/Visible detector was employed.

#### *Instrumental conditions*

To separate [<sup>3</sup>H]<sub>2</sub>-cortisone from [<sup>3</sup>H]<sub>2</sub>-cortisol a Sunfire C18 column (5µm, 15cm, 4.6mm, 35°C) was used and a mobile phase of water: acetonitrile: methanol, 60:15:25 (1.0ml/min).

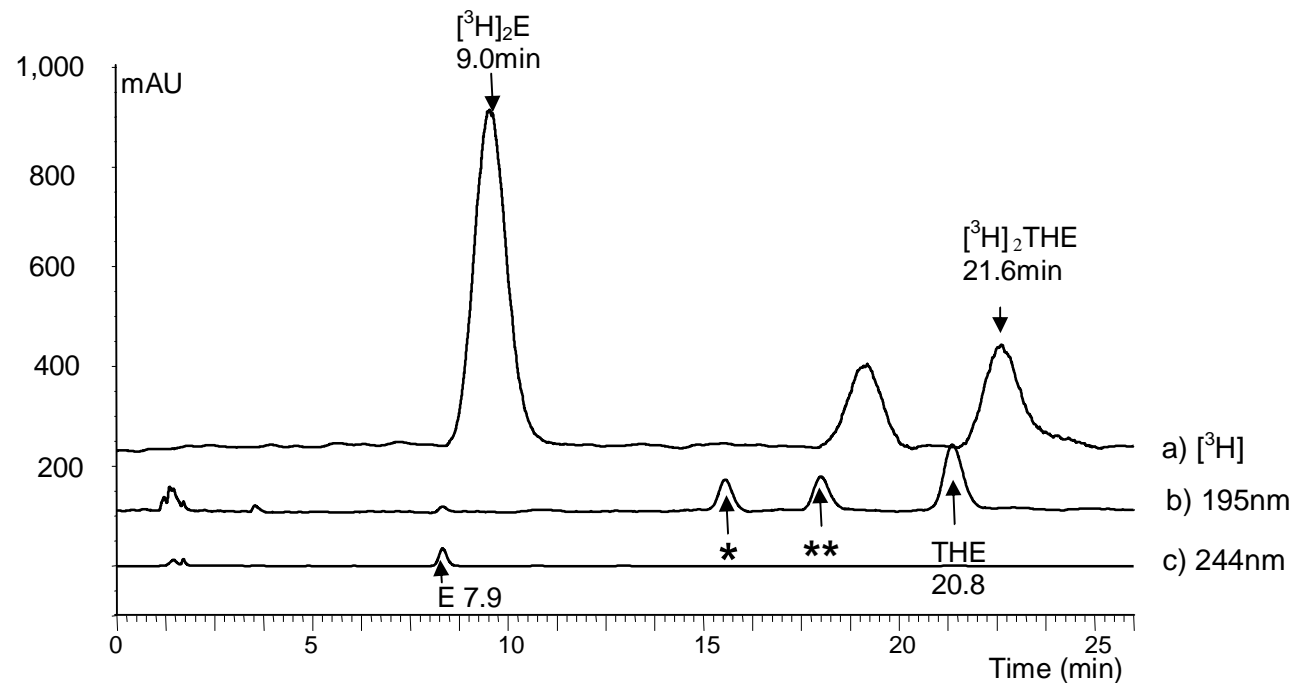
Approximate retention times for [ $^3\text{H}$ ]<sub>2</sub>-cortisone and [ $^3\text{H}$ ]<sub>2</sub>-cortisol were 19.0 and 21.8 mins respectively. To achieve optimal mixing and counting efficiency the scintillant flow rate (ProFlow G; Biotechnologies Ltd, Epsom, UK) was 2.0ml/ min.

To detect [ $^3\text{H}$ ]<sub>2</sub>-cortisone and [ $^3\text{H}$ ]<sub>2</sub>-tetrahydrocortisone the above column, mobile phase and scintillant were used, except the column temperature was 45°C and mobile phase flow rate 1.5ml/min. Approximate retention times for [ $^3\text{H}$ ]<sub>2</sub>-cortisone and [ $^3\text{H}$ ]<sub>2</sub>- tetrahydrocortisone were 9.0 and 21.6 mins respectively. Products formed from [ $^3\text{H}$ ]<sub>2</sub>-cortisone were identified by comparison of their retention times with those of unlabelled standards detected by absorbance at  $\lambda$  244nm for cortisone and 195nm for reduced metabolites. Figure 2.1 shows representative chromatograms.

### ***Data analysis***

Following chromatography, the area under each peak was integrated using the Chromeleon software (version 6.8, Dionex, Leeds, UK) and the percentage conversion of [ $^3\text{H}$ ]-substrate to [ $^3\text{H}$ ]-product quantified and converted into substrate and product concentrations. Peak areas were integrated when the response had a signal to noise ratio (SNR) >3. Data points were accepted if coefficients of variation between duplicates was <10%.

**Figure 2.1** Representative radioactive (a) and UV (b & c) chromatograms of the products of metabolism of cortisone or [ $^3\text{H}$ ]<sub>2</sub>-cortisone by human hepatic cytosol, assessing velocity of metabolism by 5 $\beta$ -reductase followed by 3 $\alpha$ -hydroxysteroid dehydrogenase



Human hepatic cytosol preparations were incubated with cortisone or d2-cortisone (10 $\mu\text{M}$ ) each with [ $^3\text{H}$ ]<sub>2</sub>-cortisone (10nM). The upper chromatogram displays the tritiated substrate and products obtained during the reaction (top trace (a)). The lower traces (b and c) are those of unlabelled steroidal standards detected by absorbance ((b) 195nm; (c) 244nm) using UV/Visible detector. [ $^3\text{H}$ ]<sub>2</sub>E= tritiated cortisone, [ $^3\text{H}$ ]<sub>2</sub>THE = tritiated tetrahydro-cortisone, E= cortisone, THE= tetrahydrocortisone, min=minutes, mAU= milli-absorbance unit; \* =  $\alpha$ -cortolone with a retention time of 15.0 mins; \*\*=  $\beta$ -cortolone with a retention time of 17.6 mins.

## 2.8.2 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

### *Instrument*

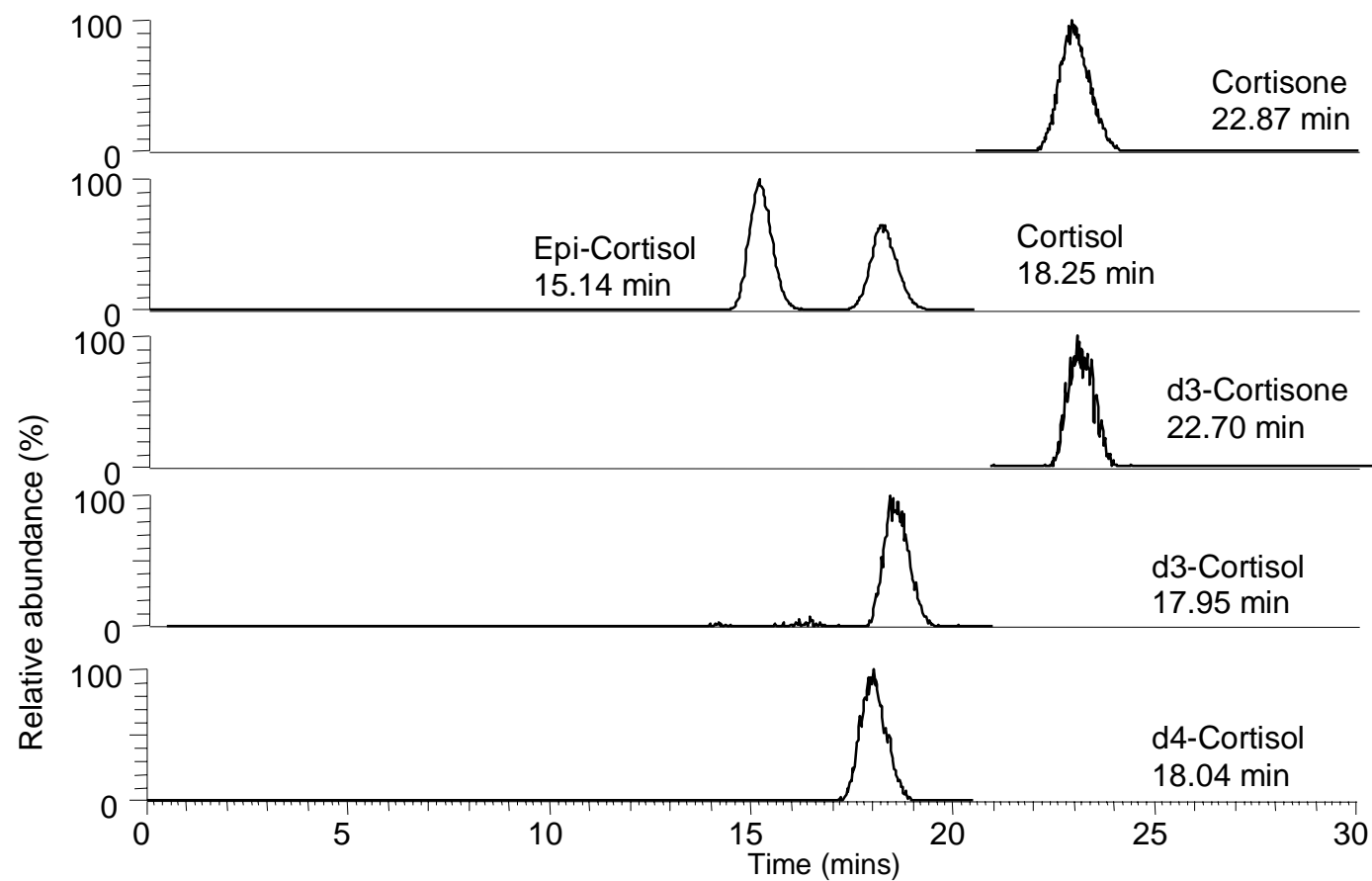
Steroids were analysed using a TSQ Quantum Discovery Mass Spectrometer and Surveyor Liquid Chromatogram (Thermo, Hemel Hempstead, UK).

### *Run conditions*

Chromatographic separation of unlabelled and deuterated cortisol and cortisone was optimised using a Biphenyl Allure column (5 $\mu$ m, 10cm, 4.6mm, 38°C (Restek, Saunderton, UK)). A mobile phase of methanol: ammonium acetate (5mM) (60:40 v/v, isocratic 0.5ml/min) was used in combination with plasma, adipose tissue, infusates and cell medium extracts. Figure 2.2 shows a representative chromatogram of steroids extracted from human plasma.

To measure cortisone and cortisol in salivary samples, the mobile phase used was methanol (+ 0.1% v/v formic acid) and water (+ 0.1% v/v formic acid) (45:55 ratio, v/v) flowing through a Kinetex PFP column (2.6 $\mu$ m, 10cm, 3.0mm, 25°C, 0.5ml/min; (Phenomenex, Torrance, CA).

Ionisation was performed in positive electrospray mode, and mass transitions of protonated ions determined using tandem MS (spray voltage 3.25kV; source temperature 400°C; collision gas pressure 1.5mTorr). Table 2.2 displays the mass transitions monitored.



**Figure 2.2** Mass chromatograms of ionised steroids extracted from human plasma

Mass chromatograms displaying peaks eluting with retention times and mass transitions indicative of cortisone  $m/z$  361 $\rightarrow$ 163, cortisol  $m/z$  363 $\rightarrow$ 121, epi-cortisol (internal standard,  $m/z$  363 $\rightarrow$ 121), d3-cortisone  $m/z$  364 $\rightarrow$ 164, d3-cortisol  $m/z$  366 $\rightarrow$ 121, and d4-cortisol  $m/z$  367 $\rightarrow$ 121 extracted from human plasma and analysed by LC-MS/MS.

**Table 2.2 Mass spectrometric conditions used for LC-MS/MS analysis**

<b>Steroid</b>	<b>Mass transitions of protonated ion <i>m/z</i></b>	<b>Tube lens (V)</b>
Cortisone	361→163	168
d2-Cortisone	363→165	118
d3-Cortisone	364→164	168
Cortisol	363→121	142
d2-Cortisol	365→122	118
	363→329	
d3-Cortisol	366→121	142
d4-Cortisol	367→121	142
Epi-cortisol	363→121	142

d=deuterium;  $m/z$  = mass charge ratio; V=volts.

### ***Data analysis***

Compounds were quantified using the ratio of (area under the peak of interest) / (area under internal standard (epi-cortisol)) using Xcalibur Quan Browser software. The standard curve was derived from the amount of steroid (x-axis) plotted against the peak area ratio (y axis). A line of best fit was drawn in the form  $y=mx+c$ . Weighting was adjusted if necessary using the software (as specified in each chapter) to optimise reproducibility of quantitation of low concentrations. The ratios obtained for steroids in the analytical sample were calculated and the amount of steroid in the sample derived from a standard curve. An enrichment curve was plotted to determine the efficiency of ionisation of the deuterated steroids compared to that of endogenous steroids. For each point on the enrichment curve, the predicted enrichment (x-axis) was plotted against the measured enrichment (y axis). A line of best fit was drawn as above and individual data points interpolated.

### ***Quality control***

Linearity was assessed by creating a standard curve representing amounts over a wide range of physiological relevance as per section 2.6.3 and assessing the regression co-efficient ( $r^2$ ) values to ensure  $>0.99$ .

As the LCMS assay for d2-cortisone was newly developed during this PhD, accuracy (the closeness of the result to the true value) and precision (how close the results are to one another) of analysis of d2-cortisone were assessed at the lowest (2.5ng) and highest (200ng) concentrations of the standard curves, weighted for  $1/\chi^2$ , and the relative mean error (RME, accuracy) and relative standard deviation (RSD, precision) calculated from  $n=6$  replicates using the equations below (de Bievre *et al*, 1998). The limit of quantification (LOQ) was determined to be the level where RSD and RME was  $<10\%$ . The limit of detection (LOD)



was defined as the lowest concentration of d2-cortisone detectable with a signal to noise ratio >3. Inter- and intra-assay variability were tested using this method.

Accuracy

or

$$\text{RME} = \frac{\text{Amount of d2-cortisone measured (ng)} - \text{Amount of d2-cortisone added (ng)}}{\text{Amount of d2-cortisone added (ng)}} \cdot 100$$

Precision

or

$$\text{RSD} = (\text{Standard deviation of mean} / \text{Mean amount of d2-cortisone detected (ng)}) \cdot 100$$

### **2.8.3 Liquid chromatography Fourier-transform mass spectrometry (LC-FT-MS)**

#### ***Instrument***

The identities of the fragment ions used for quantitative analysis were confirmed for analytical standards by liquid chromatography Fourier Transform-mass spectrometry (FT-MS) using an LTQ Orbitrap instrument (ThermoScientific, Bremen, Germany).

#### ***Run conditions***

Following elution from a Hypersil C18 column (Thermo Scientific, Hemel Hempstead, UK) (5µm, 5cm, 2.1mm, room temperature, mobile phase acetonitrile and water with formic acid (0.1% v/v) (90:10), at a flow rate of 250µl/min), compounds were subject to monitoring in full scan mode using collision-induced dissociation (CID) (45V). Masses of resultant ions were monitored in the range  $m/z$  150-1000 amu (spray voltage 4.50kV; capillary temperature

250°C). A secondary ion dependent scan was employed at 45% CID to assess fragmentation product ions.

### ***Data analysis***

Xcalibur software was used to derive the chemical formulae of the compounds of interest using the exact masses derived during LC-FT-MS.

## **2.8.4 Gas chromatography mass spectrometry (GC-MS)**

### ***Instruments***

a) Analysis of urinary and adipose steroids and products of 5 $\beta$ -reductase bioassays was achieved using a Polaris Q-Trace GC-MS (Thermo, Hemel Hempstead, UK).

b) Analysis of d2-cortisone for impurities was achieved using a Finnigan Voyager GC-MS system (Thermo, Hemel Hempstead, UK).

### ***Run conditions***

a) Urinary steroids and adipose steroids were separated using a RTX5MS column (30m, 0.25mm id, 0.25 $\mu$ m (J&W Scientific, Folsom, CA)). Ionization was performed in electron impact mode (EI 70eV). For urinary steroids, the initial temperature was 50°C (maintained for 1 minute and increased by 30°C/minute to 200°C, and then by 5°C/minute to 300°C and maintained for 5 minutes). Helium flow was 1ml/minute with source inlet and transfer line temperatures of 240°C and 280°C respectively. For steroids derived from adipose, the initial

temperature was 150°C (maintained for 1 minute and increased by 30°C/minute to 200°C, and then by 8°C/minute to 300°C and maintained for 6 minutes). Other conditions were as above. Table 2.3 shows ions monitored by selective ion monitoring.

b) For d2-Cortisone a full scan (280-700amu) was executed following separation on a DB5MS column (30m, 0.25mm, 0.5µm) (J&W Scientific, Folsom, CA). Ionization was performed in EI mode (70eV). The initial temperature was 50°C (maintained for 1 minute and increased by 30°C/minute to 200°C, then by 8°C / minute to 250°C, and then by 6°C / minute to 300°C and maintained for 10 minutes). Helium flow was 1.5ml/minute with source inlet and GC interface temperatures of 200°C and 280°C respectively.

#### ***Data analysis***

Xcalibur software was used to quantify compounds of interest.

**Table 2.3 Mass transitions monitored during GC-MS**

Steroid	Ions monitored <i>m/z</i>
5 $\beta$ -Dihydrocortisone	533
3 $\alpha$ 5 $\beta$ -Tetrahydrocortisone	578
3 $\alpha$ 5 $\beta$ - Tetrahydrocortisol	652
$\alpha$ - and $\beta$ -Cortolone	447
Cortisol	605
Cortisone	531
Epi-cortisol	605
Epi-tetrahydrocortisol	652
3 $\alpha$ ,5 $\beta$ - and 5 $\alpha$ -Tetrahydrocortisol	652
3 $\alpha$ , 5 $\beta$ -Tetrahydrocortisone	581
Epi-corticosterone	548
Epi-tetrahydrocorticosterone	564
3 $\alpha$ ,5 $\alpha$ - Tetrahydrocorticosterone	564
d2-Cortisone	533

d=deuterium; *m/z* = mass charge ratio.

## 2.9 Statistical and kinetic analysis

Equations and calculations used in tracer analysis are detailed in each chapter. The software used for kinetic analysis was Kinetica (Thermo Scientific, Philadelphia, PA). Calculations were performed in Microsoft Excel and SPSS version 14 (Chicago, IL).

All values are expressed as mean  $\pm$  standard error of the mean. Data were analysed using paired or single sample t-tests. Significance was taken at the conventional level  $p \leq 0.05$ .

In Chapter 4, all *in vitro* and *in vivo* data were  $n=3$ . As with all very small samples a normal distribution of the differences had to be assumed as neither graphical nor formal tests are useful to detect departures from normality, and non-parametric methods based on rank e.g. Wilcoxon Matched Pairs test are fundamentally incapable of detecting statistically significant differences at the 0.05 level with sample sizes of less than six (Bland, 1995).

Graphpad software was used to calculate enzyme kinetics ( $K_m$  and  $V_{max}$ ) after input of experimental data in to a pre-set template.

## **Chapter 3**

**Local versus systemic (HPA axis) cortisol  
generation- tissue tracer enrichment study**

Despite the literature describing subcutaneous adipose 11 $\beta$ HSD1 being up-regulated in obesity / metabolic syndrome, absolute levels of glucocorticoids in adipose have not been accurately quantified nor the contribution of 11 $\beta$ HSD1 to the intra-adipose glucocorticoid pool. This chapter addresses aims 1-3 from Chapter 1 Introduction: to develop a method to measure adipose tissue glucocorticoids using LC-MS/MS; to assess glucocorticoid uptake in adipose tissue using the stable isotope tracer d4-cortisol; and to determine the relative contribution of the HPA axis and adipose 11 $\beta$ HSD1 to the adipose glucocorticoid pool.

### 3.1 Introduction

Human adipose tissue contains a glucocorticoid pool derived from both the systemic circulation and from local regeneration of cortisol by the intracellular enzyme 11 $\beta$ HSD1 (Sandeep *et al*, 2005;Stimson *et al*, 2009). Intra-adipose cortisol is physiologically important as it interacts with cytoplasmic glucocorticoid receptors to regulate gene transcription, eg of metabolic enzymes such as hormone sensitive lipase (Slavin *et al*, 1994), and influence the balance of pre-adipocyte proliferation and differentiation (Bujalska *et al*, 1999). Acute elevations in plasma cortisol *in vivo* in humans have been associated with increased lipolysis in adipose tissue (Djurhuus *et al*, 2002), although results are inconsistent (Samra *et al*, 1998) and vary according to the duration of glucocorticoid exposure (Gravholt *et al*, 2002). Longer-term glucocorticoid excess is associated with adipose lipid accumulation (Macfarlane *et al*, 2008).

Concentrations of glucocorticoids in human subcutaneous adipose tissue biopsies have been estimated using several bio-analytical techniques with contrasting results (Feher & Bodrogi, 1982;Lindsay *et al*, 2003;Ronquist-Nii & Edlund, 2005;Wake *et al*, 2003). Using highly specific and sensitive LC-MS/MS, adipose tissue cortisol has been reported as 12.4 $\pm$ 0.6 and

cortisone  $3.0 \pm 0.3$  nmol/kg (Ronquist-Nii & Edlund, 2005). However, these methods do not measure the dynamics of uptake of cortisol by adipose tissue, which may explain discrepancies between effects after different durations of exposure. Traditionally, steroid transport across the cell membrane was assumed to be a passive process following a concentration gradient. However, an increasing number of transporters of steroids have been identified (section 1.1.1). Moreover, measurement of concentration within adipose does not distinguish cortisol derived from the systemic circulation from that generated locally by 11 $\beta$ HSD1 (Sandeep *et al*, 2005;Stimson *et al*, 2009), which may be important in determining glucocorticoid activity in human obesity (Rask *et al*, 2001;Wake *et al*, 2003) and is a target for pharmacological inhibition in diabetes (Hughes *et al*, 2008).

Dynamic turnover in the intra-adipose cortisol pool can be investigated using a stable isotope tracer such as 9,11,12,12-[<sup>2</sup>H]<sub>4</sub>-cortisol (d4-cortisol) (section 1.11), which allows quantification of cortisol production in the plasma (Andrew *et al*, 2002) and across tissue beds using arterio-venous sampling (Stimson *et al*, 2009).

## **3.2 Aim**

To develop a protocol for efficient extraction of glucocorticoids from human adipose tissue biopsies, and study the incorporation and dilution of d4-cortisol tracer in adipose tissue during intravenous steady state infusion. This will allow calculation of the rate of cortisol uptake in adipose tissue and assessment of the amount of cortisol generated locally in adipose compared to the amount derived from the systemic circulation.



### **3.3 Methods**

#### **3.3.1 Study Design**

The study was approved by the Local Research Ethics Committee and all subjects gave written informed consent.

##### ***Subjects***

To assess adipose cortisol dynamics, healthy female volunteers (n=6), who had not received glucocorticoid treatment by any route for 3 months, with normal haematological and renal indices, who were undergoing an elective abdominal hysterectomy for benign gynaecological indications attended the Clinical Research Facility between 08:30-10:30h after an overnight fast. Subjects were studied once in the Royal Infirmary of Edinburgh on the day of their elective operation.

At the beginning of recruitment we aimed to assess individuals naive to any medication. We were unable to recruit the required number of subjects using this strategy as most were taking analgesia or taking part in other clinical research studies. By changing our recruitment strategy and accepting volunteers on some medications (known not to interfere with glucocorticoid metabolism) we were able to study all volunteers from the gynaecology clinics.

##### ***Clinical measurements***

Height, weight (clothed), waist and hip circumference were measured using standard techniques. For height, volunteers stood without shoes against a fixed measuring ruler on a

wall and the measurement at the top of the head recorded in metres (to 2 decimal places). Weight was measured in kilogrammes on an electronic scale (Seca, Birmingham, UK) after ensuring the instrument was calibrated. For waist: hip ratio (WHR), waist circumference was measured midway between the lower rib margin and the iliac crest with the volunteer standing with their hands by their sides, using a non-expandable measuring tape (Lufkin executive W606P). Hip circumference was taken with the subject in the same position, by wrapping the tape measure around the buttocks and the maximum circumference located. Body fat percentage was taken as the average of two consecutive readings, using an OMRON BF306 Body Fat Monitor (OMRON Healthcare (UK) Ltd, Henfield, UK) after an overnight fast. The monitor was pre-set to the correct height, weight, age and gender with the results obtained on the morning of the study. Volunteers stood with their feet apart and their arms at 90° to their trunk, with the palm of each hand placed firmly on both electrode handles. Blood pressure was measured after sitting for at least 10 minutes using a 705IT automatic blood pressure monitor (OMRON Healthcare Europe BV, Hoofddorp, NL).

***Preparation of the stable isotope tracer (60% hydrocortisone and 40% d4-cortisol)***

To prepare the isotope for this study, unlabelled hydrocortisone (12mg) and d4-cortisol (8mg) were dissolved in pharmaceutical grade ethanol (2.5ml) and filtered in the radio pharmacy to achieve sterility. From this stock solution, 0.5ml was dissolved in sodium chloride 0.9% w/v (60ml) immediately prior to use to give a solution with a concentration of 0.067mg/ml. 7.5ml of this solution was discarded, leaving 52.5ml (containing 3.5mg total steroid (cortisol + d4-cortisol)) for use as the bolus injection.

For an intravenous infusion, 1.5ml (containing 12mg total steroid) of stock solution was diluted in sodium chloride 0.9% w/v (500ml) immediately prior to use to achieve a solution

with a final concentration 0.024mg/ml. This was infused at a rate of 72.5ml/hour to deliver 1.74mg of hydrocortisone and d4-cortisol (60:40 ratio) per hour.

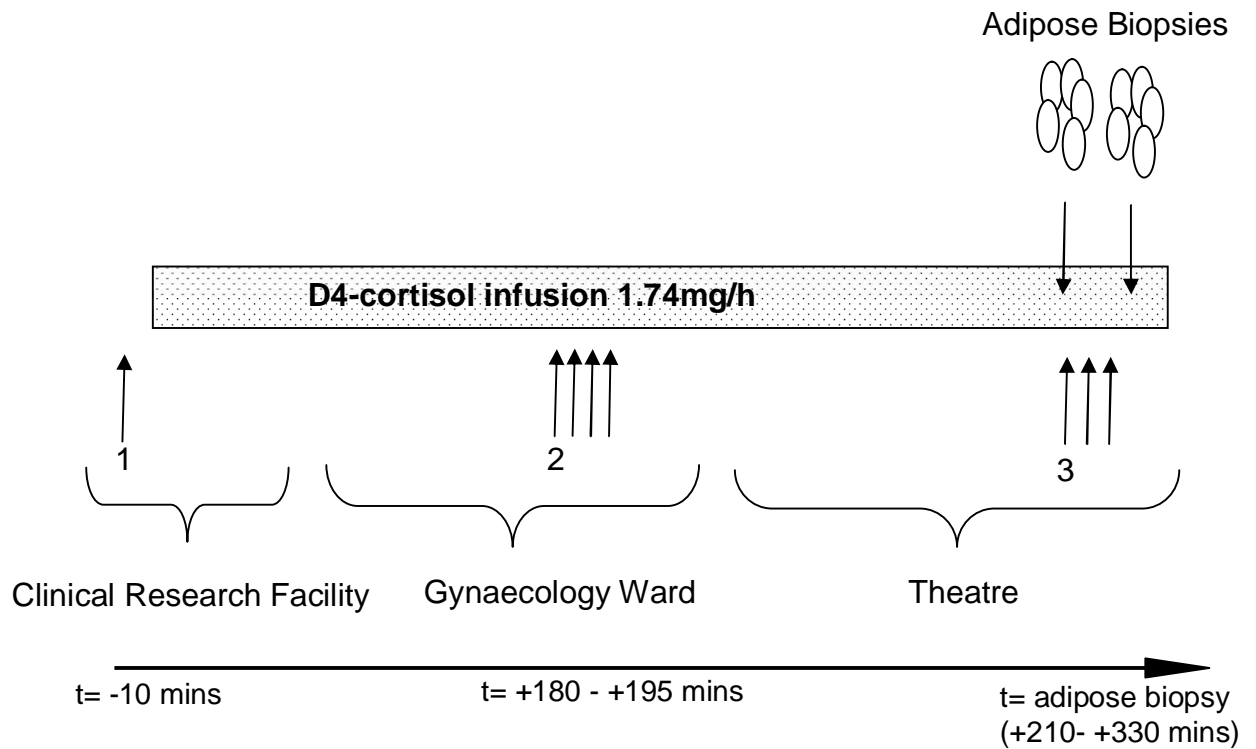
### ***Clinical protocol***

One intravenous cannula was inserted into each antecubital fossa vein. Venous blood was collected for basal assessment of steroids and background isotopomers (Figure 3.1). An intravenous bolus of d4-cortisol was administered (3.5mg) over 5 minutes, followed by a continuous intravenous infusion (1.74mg/hour). Four venous blood samples were obtained at five minute intervals after 3 hours, when plasma steady state was achieved from the arm contra-lateral to the infusion (Andrew *et al*, 2002). Anaesthetists were asked to withhold medication that could interfere with endogenous glucocorticoid metabolism (e.g intravenous dexamethasone for post-operative anaesthesia-related vomiting) until after the study was complete. Study participants were then transferred to theatre with the d4-cortisol infusion running, where both subcutaneous and omental adipose tissue samples were obtained by the surgeon upon opening the abdomen. Three venous blood samples were taken at 5 minute intervals at the time of the subcutaneous adipose biopsy from the anterior abdominal wall to measure concurrent enrichment of plasma and adipose with d4-cortisol.

### ***Sample collection and processing***

Prior to obtaining any blood samples, 2ml of dead space / waste (a mixture of saline and blood in the cannula) was withdrawn from the sampling cannula to prevent contamination of the whole blood sample by saline. Whole blood samples were obtained in 5.5ml lithium heparin S-Monovette tubes (Starstedt, Beaumont Leys, UK). Blood samples were placed on wet ice prior to centrifugation (3000 x g 10min, 4°C) to obtain plasma and stored at -20° C.

Adipose samples were collected in conical bottom falcon tubes (50ml) without any preservative, snap frozen on dry ice and stored at  $-80^{\circ}\text{C}$  prior to analysis. A sample of d4-cortisol infusate was collected at the end of the study and stored at  $-20^{\circ}\text{C}$ .



**Figure 3.1: Study protocol.**

d4-Cortisol infusion was infused intravenously at 1.74mg / hour and commenced after obtaining venous blood for basal isotopomers. Arrows indicate blood sampling, (1) for basal isotopomers, (2) at steady state,  $t = +180 - t = +195$ , (3) time of the first adipose biopsy at 5 minute intervals.

### 3.3.2 Extraction of adipose tissue glucocorticoids

#### *Sources of adipose tissue for assay development*

For assay development adipose tissue from a male Zucker rat, (593g body weight) was used. The animal had received a subcutaneous bolus injection of d4-cortisol (250µg in 0.9% saline: ethanol (95:5 v/v) (0.5ml)) an hour prior to sacrifice. Adipose depots were snap frozen on dry ice and stored at -80°C until analysis.

Human adipose tissue was obtained surgically from this clinical study. A second source of human subcutaneous adipose tissue was from archived tissue samples obtained by needle biopsy more than 5 years previously (Wake *et al*, 2003).

#### *Method development*

The initial extraction method had been developed in-house and consisted of 4 main stages: a) splitting adipocytes to release intracellular glucocorticoids, b) cleaning the extract and removing oils, c) re-extracting glucocorticoids and d) derivatisation prior to analysis by GC-MS. The quantity of steroid recovered was determined as a ratio to a known amount of added internal standard, and percentage recovery assessed on an un-extracted standard. At each step, alternative processing methods were compared and the protocol yielding the maximal recovery and best signal to noise ratio taken forward. All methods were performed in duplicate or triplicate at least one time.

*a) Splitting adipocytes*

To split adipocytes and release intracellular glucocorticoid, adipose tissue (250mg) was cut into small pieces on a bed of dry ice and weighed in a glass dish. The tissue was transferred into a glass tube and sodium phosphate buffer (section 2.3.1a) added (1ml). Tissues were stored on dry ice before being subjected to 3x 10 seconds bursts of mechanical homogenisation (in a class 1 bio-safety cabinet when human tissues used). Samples were placed on dry ice for 30 seconds in-between bursts of homogenisation. Internal standards (1µg, epi-corticosterone and epi-tetrahydrocorticosterone for rodent tissues, epi-cortisol for human tissue) were added and the homogenate dripped onto glacial acetic acid: ethanol: water (3:95:2ml v/v, 10ml, chilled on dry ice) using a glass pipette. The homogenate remained in acetic acid-ethanol mix overnight (-80°C). Samples were placed on wet ice (30 mins), before being subjected to sonication (8 x 15 sec bursts, placing on wet ice for 1 min between bursts) and centrifugation (5900 x g, 30 min, room temp) to separate the cell membranes from lighter cellular organelles. The supernatant was transferred to a clean tube, reduced to dryness (OFN, 60°C) and further methanol added (10ml), before storing the samples overnight (-80°C).

To optimise the homogenisation step, sodium phosphate buffer stored at room temperature (section 2.3.1a) (1ml), fresh sodium phosphate buffer solution (section 2.3.1a) (1ml), HPLC-grade water (1ml), and ethyl acetate (1ml) were tested as the homogenisation fluid. To minimise adsorption of endogenous glucocorticoids to centrifuge containers both plastic and glass centrifuge tubes were also assessed.

*b) Cleaning the extract and removing oils*

The samples attained room temperature and hexane (10mls) added and mixed. The hexane layer was discarded and the remaining methanol transferred into a clean glass tube and reduced to dryness (OFN, 60°C). This step was not altered.

*c) Re-extracting glucocorticoids*

Water (400µl) and ethyl acetate (4ml) were added to the extract and mixed. The organic layer was removed and reduced to dryness in glass vials. The re-extraction step was not altered, but prior to derivatisation for GC-MS an extra cleaning step was added to remove oils.

To optimise removing oils, Lipidex 5000 mobile phase (1ml) (section 2.7.1), methanol (5ml) and 30% methanol (5ml) were assessed as solvents for dissolving the dried adipose extract prior to applying the extracts to 3 different types of column. The columns assessed were a Lipidex 5000 column (section 2.7.2), a Sep-Pak C18 column (section 2.6.2), and a C18 Bond Elut column (section 2.6.6). The solvents assessed for eluting the steroid from the column were water (2ml), 50% methanol (2ml), 100% methanol (2-5ml), acetonitrile (2ml), ethyl acetate (2-5ml) and hexane (2ml).

*d) Derivatisation prior to analysis by GC-MS*

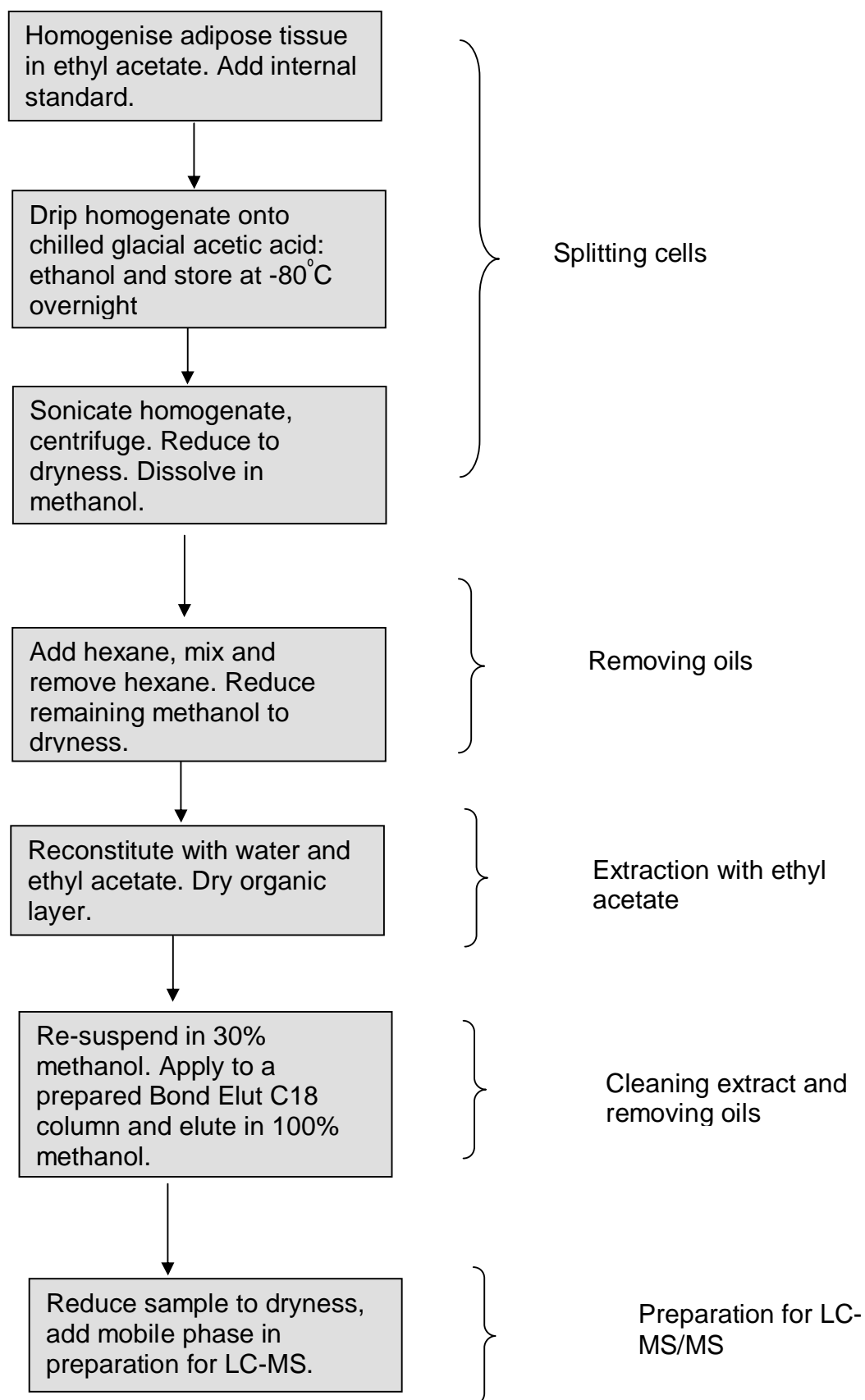
The final step was derivatisation prior to GCMS. LC-MS/MS analysis was also assessed when human steroids and samples were used, in which case derivatisation was not necessary.

### ***Final method***

The final method used to extract adipose glucocorticoids is detailed in section 2.6.6. Please see Figure 3.2 for a summary of stages of the extraction process. Paired human subcutaneous and omental adipose samples were analysed in duplicate.



**Figure 3.2 Adipose tissue glucocorticoid extraction method**



### 3.3.3 Extraction of plasma glucocorticoids

Endogenous and deuterated glucocorticoids were extracted from plasma using the method detailed in section 2.6.3.

### 3.3.4 Analytical techniques

Plasma and adipose tissue steroidal extracts were analysed using LC-MS/MS as per section 2.8.2.

### 3.3.5 Data analysis

The peak area of d4-cortisol was corrected for interference from the m+4 isotopomer of cortisol and the m+1 isotopomer of d3-cortisol. The peak area of d3-cortisol was corrected for the m+3 isotopomer of cortisol and d3-cortisol signal in d4-cortisol. *Equations 1 & 2* were solved as simultaneous equations. Concentrations of cortisol and cortisone were determined from a standard curve. Calibration and enrichment curves were prepared for both plasma and adipose assays.

#### *Plasma*

Plasma d3-cortisol and d4-cortisol concentrations values were calculated by multiplying the concentrations of cortisol by their respective tracer:tracee ratios (*Equation 3*). Rate of appearance of cortisol and d3-cortisol in the plasma were calculated as per *Equations 4 & 5* (Stimson *et al*, 2009). Pre-operative data were calculated from the mean results in 4 samples obtained from each participant between 180-195 minutes of d4-cortisol infusion and intra-

operative data from the mean results of 3 samples obtained after a variable period of infusion (234-331 min).

*Equation 1:*

$$\text{Peak area d3-cortisol} = \text{true area d3-cortisol} + 6.8\% \text{ of peak area d4-cortisol}$$

*Equation 2:*

$$\text{Peak area d4-cortisol} = \text{true d4-cortisol} + 15\% \text{ of peak area d3-cortisol}$$

*Equation 3:*

$$\begin{aligned} \text{Concentration at steady state (C}_{ss}\text{) d3-cortisol or d4-cortisol (nM)} \\ = [\text{cortisol}]_{\text{nM}} \cdot \text{Tracer:Tracee (TTR)} \end{aligned}$$

*Equation 4:*

$$\begin{aligned} \text{Ra-cortisol (nmol/min)} = \\ (\text{Rate of d4-cortisol infused} / \text{TTR(d4-cortisol: cortisol)}) - \text{Rate of cortisol infused} \end{aligned}$$

*Equation 5:*

$$\text{Ra d3-cortisol} = \text{Rate of d4-cortisol infused} / \text{TTR (d4-cortisol:d3-cortisol)}$$

### ***Adipose tissue***

For adipose tissue extracts, the limit of quantitation for the LC-MS/MS was assigned with a signal: noise ratio >3 and peaks underwent smoothing (x5). Amounts of cortisol and cortisone were determined from a standard curve. Adipose tissue d3-cortisol, d3-cortisone and d4-cortisol amounts (nmol/kg of adipose) were calculated using the calculated amount of the non-deuterated glucocorticoids multiplied by the respective tracer:tracee ratios. All

adipose tissue amounts in mass/kg were converted to concentrations in mass/l by multiplying by the relative density of fat of 0.9g/ml (Ross *et al*, 1991).

The rate of accumulation of d4-cortisol in adipose tissue was calculated using *Equation 6*, expressed as nmol/kg/h, assuming that no d4-cortisol was present in the adipose tissue before infusion, and that the rate of accumulation of tracer in adipose tissue was approximately linear during infusion.

*Equation 6*

Rate of net d4-cortisol uptake into adipose (nmol/kg/h) = [d4-cortisol]<sub>adipose</sub> / time to biopsy

The proportion of the intra-adipose cortisol pool that was replaced during d4-cortisol infusion was calculated using *Equation 7*, and expressed as %/h.

*Equation 7*

Proportion of adipose cortisol pool

replaced by d4-cortisol

$$(\%/h) = \left( \frac{\left( \frac{(\text{d4-cortisol}/(\text{d4-cortisol} + \text{cortisol}))_{\text{adipose}}}{(\text{d4-cortisol}/(\text{d4-cortisol} + \text{cortisol}))_{\text{plasma}}} \right) / \text{time to biopsy}}{1} \right) \cdot 100$$

Calculations were performed in Microsoft Excel and SPSS version 14 (Chicago, IL). Values are expressed as mean  $\pm$  SEM. Data were analysed using Student's t-tests. Significance was taken at  $p < 0.05$ .

## 3.4 Results

### 3.4.1 Adipose tissue method development

The initial extraction method consisted of 4 main stages: a) splitting adipocytes to release intracellular glucocorticoids, b) cleaning the extract and removing oils, c) re-extracting glucocorticoids and d) derivatisation prior to analysis by GC-MS.

Using this initial method recovery of internal standard was poor and inconsistent with rodent adipose tissue. The method was repeated without adipose extract ie with reagents only to assess recovery of added internal standard. Upon analysis, the GC-MS traces had a high signal to noise ratio and a low steroid recovery (<1-1.3%). To optimise the method and assess where the internal standard was being lost, the method was assessed in a stepwise fashion and divided into the 4 key steps described above. These stages were assessed individually by commencing the process at the sequential point with reagents only and internal standard (ie without adipose initially) and analysed by GC-MS.

Starting with the last stages of the extraction method, the derivatisation, re-extraction with ethyl acetate, and removal of oils stages were all successful but gave variable recovery of internal standard (10-71%).

It was noted that the sodium salts of the buffer solution used for homogenisation precipitated when added to the chilled ethanol: acetic acid mixture. To assess if the precipitated buffer solution or indeed debris in an older solution (stored at room temperature) was the cause of the poor GC-MS traces, the splitting cells step was further divided into 2 stages and assessed. Addition of the buffer led to lower internal standard recovery (15-20%) and interference on

the GC-MS trace with a high signal to noise ratio, compared to the stage without buffer (ethanol: acetic acid mixture alone).

The splitting cells step was repeated with either fresh buffer solution or water (no adipose). Greater steroid recovery was achieved at this step with fresh buffer compared to water (>100% vs 16- 36%). The whole method (without adipose) was repeated with the freshly made sodium phosphate buffer. A small amount of internal standard was recovered (5-37%).

As we had demonstrated that during each of the steps with reagents only, a variable amount of internal standard could be recovered; the whole method was repeated with adipose tissue and broken into 4 steps as above. Despite using fresh buffer solutions, recovery of added internal standard remained poor (recovery 1-17%).

### ***Optimising the homogenisation fluid***

As the homogenisation buffer had initially been problematic, ethyl acetate was assessed as a substitute (Ronquist-Nii & Edlund, 2005). When comparing fresh buffer to ethyl acetate for the homogenisation step, (the methods were assessed in parallel without adipose), recovery of added internal standards were comparable (76-100% for ethyl acetate vs 78-89% with buffer). However, the GC-MS traces were more reproducible with the ethyl acetate, with a lower signal to noise ratio. Therefore, ethyl acetate was chosen as the homogenisation fluid.

Using ethyl acetate, the whole method was repeated with adipose. The centrifugation step was modified to allow the use glass tubes in the centrifuge (3000 x g, 30 min, 4°C). This modification was to prevent endogenous or added glucocorticoids sticking to plastics in the centrifugation tubes thus lowering recovery, as other steroids such as oestrogen and

progesterone readily stick to plastics. Recovery of internal standard was poor and varied (0.5-14%), but there was little background noise on the GC-MS trace.

### *Adding extra steps to purify the extracts*

Upon viewing the derivatised steroidal extract, they appeared oily and yellow in colour. Water is known to interfere with some derivatising agents and so it is possible that water and oils in the extract may have interfered with the derivatisation process. This may have accounted for the large variability of results in all of the previous experiments. A further purifying step was added prior to derivatisation and several purification columns assessed for this process (lipidex columns, Sep-Pak C18 columns, and Varian Bond Elut C18 columns).

The lipidex column was not successful in improving the yield of derivatised steroids and the extract remained yellow in colour. Prior to applying the adipose extracts to Sep Pak C18 columns the solvent for re-suspending the adipose extract and for eluting the glucocorticoids was optimised. This was achieved by spiking adipose extracts with tritiated glucocorticoids and assessing the recovery of tritiated steroid in the eluate from the column using a beta-counter. Initially 100% methanol (5ml) was assessed as the solution to re-suspend the adipose extract (1µl  $^3\text{H}$ -corticosterone added). Only 12% of the added tritiated steroid was recovered. On reviewing the results of the experiment, it was evident that  $^3\text{H}$ -corticosterone did not stick to the column. When the adipose extract was applied, 43% of radioactivity was lost. Further loss occurred when water was passed through the column. Therefore, to improve separation of  $^3\text{H}$ -corticosterone from adipose tissue, improve steroidal sequestration on the column and subsequent elution from the column, adipose extracts were reconstituted in a less polar solvent. Using 30% methanol (methanol: water 30:70 v/v), only 7.8% of radioactivity was lost when applying the extract to the column and a further 1.2% after

applying a wash (100% water). Therefore this was chosen as the solvent for re-suspending adipose extracts prior to using the column.

The extracts were eluted with one of the following solvents: water (2ml), 50% methanol (2ml), 100% methanol (2ml), acetonitrile (2ml), ethyl acetate (2ml) and hexane (2ml) to assess the solvent giving optimum recovery of radioactivity. Ethyl acetate was the best (>100% recovery of tritiated steroids) with 100% methanol and acetonitrile being next best (62% and 70% respectively). However, ethyl acetate appeared to melt the plastics on the columns. When the solvents from these experiments were applied to adipose extracts with non-tritiated internal standards and analysed by GC-MS, recovery of internal standards remained poor (0.3-20%) and the adipose extracts remained light yellow in colour.

C18 Bond Elut cartridges (2g, Varian, Oxford, UK) were prepared and adipose extracts (in 5ml 30% methanol) applied and the steroids eluted in ethyl acetate. After passing the adipose extract sample through the column the eluate was colourless, but recovery of steroids remained variable (1.5-40%). As the ethyl acetate used to elute steroids from the column appeared to melt the plastic column, this was substituted for methanol: ethyl acetate (50:50 v:v). With this modification the GC-MS traces improved but internal standard recovery remained low. The elution solvent was modified from methanol: ethyl acetate (50:50 v:v) to 100% methanol as we were concerned that plasticisers from the melted column and resultant may interfere with mass spectrometric analysis. Excellent recovery of  $^3\text{H}$ -glucocorticoid was achieved (86%).



### ***Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)***

After using the Varian column, the adipose steroidal extracts were colourless and oil-free. The analysis method was changed to LC-MS/MS, as the variability in the GC-MS results may have been due to inadequate derivatisation. Using LC-MS/MS, recovery of spiked internal standards (epi-cortisol, cortisol, cortisone and d4-cortisol) from rodent adipose was excellent (85->100%). The method was broken into stages and internal standard added in a stepwise fashion to assess if there was any point where recovery of steroids was sub-optimal. Each of the stages produced good added steroid recovery except the initial homogenisation step (homogenisation 46% recovery, splitting adipocytes with ethanol: acetic acid 67% recovery, sonication 67% recovery, removing oils with hexane 69% recovery, putting through columns 100% recovery).

### ***Optimising the method with human tissue***

With the method giving 85->100% recovery of added internal standard with rodent adipose tissue, the method was assessed with human adipose tissue. Cortisone concentrations in subcutaneous adipose were 11-19.5nmol/kg and 12-44nmol/kg in omental adipose. Cortisol concentrations were 16-36nmol/kg in subcutaneous and 29-47nmol/kg in omental tissue. D4-cortisol was 7-10nmol/kg in both depots and d3-cortisol levels 50-60% of the d4-cortisol values.

The technique was repeated using a larger amount of human adipose tissue to assess if this improved endogenous glucocorticoid recovery. Endogenous steroid recovery was inferior with 400mg of adipose compared with 250mg, (cortisol 6.2 vs 16.9 nmoles/kg, cortisone 3.5 vs 13.3 nmoles/kg, d4-cortisol 3.4 vs 10.2 nmoles/kg respectively). A 'null' adipose sample

(archived tissue) for assessment of background isotopomers was analysed and did not reveal any d4-cortisol isotopomers. The optimised method achieved an extraction efficiency of  $70.1 \pm 2.1\%$  for internal standard using 250mg. The RSD of triplicates was 13.4% for omental and 22.8% for subcutaneous adipose tissue. Please refer to Figure 3.3 for final experimental method.

### **3.4.2 Clinical results: samples obtained**

The intravenous infusion was well tolerated with no serious adverse events. Unforeseen problems which precluded obtaining 100% of plasma samples included malfunction of sampling cannulae during volunteer transfer. Despite this, the majority of plasma (46/48, 95.8%, n=6) samples, and all adipose tissue (12/12, 100%, n=6) samples were obtained from each study participant.

### **3.4.3 Participant characteristics**

Participants were aged  $48.3 \pm 3.8$ y, with BMI  $30.9 \pm 2.9$ kg/m<sup>2</sup>, and fat mass  $30.7 \pm 3.5$ kg (Table 3.1). All were undergoing total abdominal hysterectomy under general anaesthesia for benign gynaecological indications (5 for uterine fibroids, 1 for endometrial polyp). Two were taking progestogens (norethisterone) and one was taking oral oestradiol (Elleste Solo) with intra-uterine progestogen (levonorgestrel). Other medications included anti-hypertensives (an ACE-inhibitor, a thiazide diuretic, and a beta-blocker) (n=3), ferrous sulphate (n=2), mefenamic acid (n=1), and levo-thyroxine (n=1).

**Table 3.1 Participant characteristics.**

	<b>Age (y)</b>	<b>BMI (kg/m<sup>2</sup>)</b>	<b>WHR</b>	<b>Bio-impedance (%)</b>	<b>Fat mass (kg)</b>	<b>SBP (mmHg)</b>	<b>DBP (mmHg)</b>
Mean							
± SEM	48.3±2.7	30.9±2.1	0.9±0.1	37.1±2.3	30.7±3.5	144.3±5.5	87.7±5.2

All data are mean ± SEM for n=6. BMI= body mass index; kg/m<sup>2</sup>= kilograms per metre squared; WHR= waist-hip ratio; SBP= systolic blood pressure; DBP= diastolic blood pressure; mmHg = millimetres of mercury.

#### 3.4.4 Plasma glucocorticoids

Deuterated and endogenous cortisol isotopomers in plasma (Figure 3.3A) were in steady state by 3 hours of infusion (not shown) and were unchanged during surgery. Cortisone but not d3-cortisone concentrations decreased during surgery (Figure 3.3C).

Dilution of d4-cortisol by unlabelled cortisol (from 40% in the infusate to ~16% in plasma, Table 3.2) was used to calculate rate of appearance of cortisol, which did not differ during surgery (pre-operative  $128.3 \pm 12.2$  nmol/min versus intra-operative  $138.4 \pm 18.5$  nmol/min,  $p=0.55$ ). Dilution of d4-cortisol by d3-cortisol was used to calculate rate of appearance of d3-cortisol, which did not differ during surgery (pre-operative  $30.5 \pm 2.2$  nmol/min versus intra-operative  $30.4 \pm 3.0$  nmol/min,  $p=0.92$ ). Study infusates were extracted to confirm the infusion was prepared correctly, and the mean amount infused was  $1.67 \pm 0.28$  mg/h ( $4.6 \mu\text{mol/h}$ ); this comprised mean amounts of cortisol and d4-cortisol of  $0.80 \pm 0.13$  mg/h ( $2.2 \mu\text{mol/h}$ ) and  $0.87 \pm 0.15$  mg/h ( $2.4 \mu\text{mol/h}$ ), respectively.

**Table 3.2: Plasma and adipose tissue tracer:tracee ratios and enrichments**

	Plasma		Adipose	
	Pre-operative	Intra-operative	Subcutaneous	Omental
d4-Cortisol enrichment (%) (d4-cortisol/(cortisol +d4-cortisol))•100	15.6±0.8	15.5±1.0	7.2±0.6*	7.4±0.7*
d3-Cortisone enrichment (%) (d3-cortisone/ (cortisone +d3-cortisone)) •100	24.5±0.8	25.4±1.0	3.8±1.2**	5.1±0.9**
[d4-cortisol]: [d3-cortisol] ratio	1.1±0.1	1.1±0.1	1.0±0.1	1.0±0.1
Cortisol: d4-cortisol:d3-cortisol ratio	5.7:1:1	6.0:1:1	13.3:1.1:1	12.3:1:1

All data are mean ± SEM for n=6. d=deuterium; \*\* p<0.0001, \* p<0.001 versus intra-operative plasma values by paired Student's t-tests.

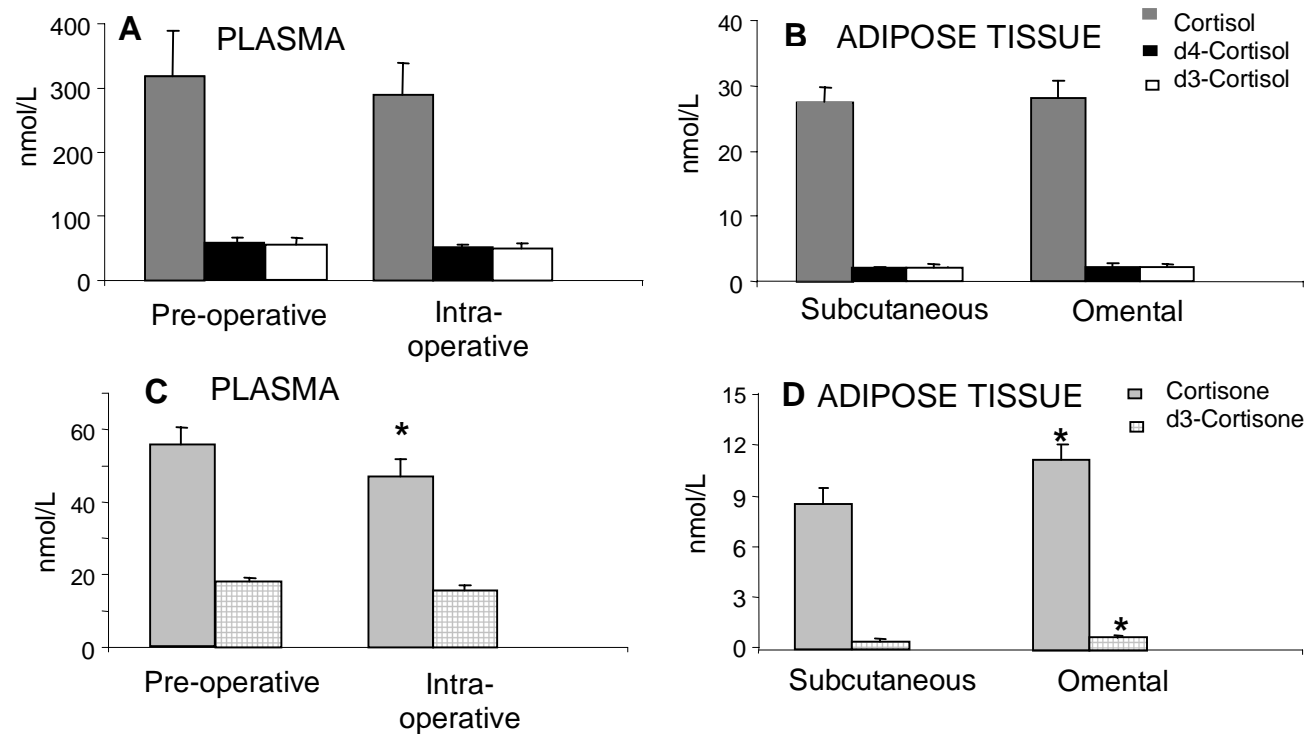
### 3.4.5 Adipose tissue glucocorticoids

Endogenous and deuterated glucocorticoids were detected in both subcutaneous and omental adipose tissue (Figures 3.3B and 3.3D) after 3.5-5.5 hours of d4-cortisol infusion. Absolute steroid concentrations were ~10-fold in both adipose tissue depots than in plasma (intra-operative plasma cortisol  $289.3 \pm 49.2$  nmol/L vs subcutaneous adipose tissue cortisol  $27.4 \pm 2.3$  nmol/L,  $p=0.003$ , intra-operative plasma cortisone  $47.2 \pm 4.7$  nmol/L vs subcutaneous adipose tissue cortisone  $8.6 \pm 1.0$  nmol/L,  $p=0.0005$ ). Cortisone and d3-cortisone concentrations were higher in the omental compared to the subcutaneous depot (Figure 3.3).

The proportions of cortisol:d4-cortisol:d3-cortisol in adipose tissue differed from those in plasma (Table 3.2). Dilution of d4-cortisol with cortisol was substantially greater in both adipose tissue depots than in plasma, but dilution of d4-cortisol with d3-cortisol was not different. Enrichment of the cortisone pool with d3-cortisone was similarly much lower in adipose tissue than in plasma.

The estimated net rate of accumulation of d4-cortisol (please see *Equation 6*) was  $0.5 \pm 0.1$  nmol/kg/h for subcutaneous and  $0.4 \pm 0.1$  nmol/kg/h for omental depots ( $p=0.73$ ) despite the 40% d4-cortisol infusion contributing  $2.4 \mu\text{mol/h}$  d4-cortisol into the circulation (see composition of extracted infusates above). The proportion of the adipose tissue cortisol pool replaced each hour was estimated as  $10.7 \pm 1.0\%$  for subcutaneous and  $10.4 \pm 0.7\%$  for omental depots ( $p=0.82$ ) (please see *Equation 7*).

**Figure 3.3: Concentrations of deuterated and endogenous cortisol and cortisone in plasma pre- and intra-operatively and in adipose depots intra-operatively.**



A) Plasma cortisol and its isotopomers (nmol/L). B) Adipose tissue cortisol and its isotopomers (nmol/L adipose tissue). C) Plasma cortisone and d3-cortisone (nmol/L). D) Adipose tissue cortisone and d3-cortisone (nmol/L adipose tissue). All data are mean  $\pm$  SEM for n=6. Pre-operative plasma values were mean of 4 samples over 15 minutes, intra-operative samples were mean of 3 samples over 10 minutes. Adipose tissue values were mean of duplicates. \*p<0.05 versus the same steroid isotopomer within each panel compared by Student's t-tests. All steroids were significantly lower in adipose tissue versus intra-operative plasma. Grey = cortisol, black = d4-cortisol, white = d3-cortisol, light grey = cortisone, grey square hatching = d3-cortisone.

### 3.5 Discussion

These data show that concentrations of deuterated cortisol and its metabolites in human adipose tissue during prolonged steady state intravenous infusion are substantially lower than the values of total cortisol (bound to CBG and free) measured in plasma. Concentrations of unlabelled cortisol and cortisone pools in adipose tissue are more than twice as high as previously reported with LC-MS/MS assays in subjects not receiving a tracer infusion (Ronquist-Nii & Edlund, 2005). There were no differences in rates of tracer accumulation or endogenous steroid levels in omental versus subcutaneous adipose depots. Thus it can be concluded that in humans *in vivo* uptake of glucocorticoids into adipose tissue and turnover of the intra-adipose glucocorticoid pool is slow. However, the contribution of 11 $\beta$ HSD1 cannot readily be assessed in this duration of study, nor the exact location of the cortisol pool in adipose tissue (eg the aqueous cytoplasm or lipid droplet).

In order to assess adipose steroid turnover, a robust method for extraction of tissue steroids was developed. Most previous studies of concentrations of steroids in adipose tissue have relied on immunoassays (Feher & Bodrogi, 1982; Lindsay *et al*, 2003; Wake *et al*, 2003) and have paid insufficient attention to matrix effects in crude tissue extracts. Others have used highly sensitive and specific LC-MS/MS (Ronquist-Nii & Edlund, 2005), reporting lower steroid concentrations than described here, although the characteristics of the participants and the biopsy method are unclear, making comparison with this study difficult. Using the knowledge gained from developing the adipose tissue glucocorticoid extraction method and the difficulties encountered, it is likely that the procedures described in the literature to extract steroids from adipose tissue are unreliable, with poor yields as considerable effort was required to optimise recovery of steroids and hence quantification.



This is the first time the 40% d4-cortisol tracer infusion has been used during surgery and in women. There was disproportionately high endogenous whole body cortisol production (~4-fold increase) but similar systemic d3-cortisol production rates as previously reported during 40% tracer infusion (30 vs 28nmol/min) (Stimson *et al*, 2009), consistent with increased adrenal secretion in association with surgery. During abdominal or hip surgery, endogenous plasma cortisol rises rapidly after the skin incision and remains elevated for >72 hours (Naito *et al*, 1992). Interestingly, this high endogenous cortisol production occurred in anticipation of surgery as well as during the procedure. In the previous study using a 40% d4-cortisol infusion the subjects were male and had received dexamethasone the evening prior to the study to suppress endogenous cortisol production (Stimson *et al*, 2009) which may partly explain some of the differences. However, gender may also play a role, in keeping with the rodent literature where baseline corticosterone levels are higher and the hypothalamic-pituitary-adrenal axis highly responsive to ACTH stimulation or stressors in females compared to males (Kitay, 1961). However in humans, gender differences in adrenocortical response to stress are more contentious, with no sexual dimorphism following either pharmacological or physical stimulation being reported, but actually a greater response in men occurs in anticipation of and during psychosocial stress (Kirschbaum *et al*, 1992).

In spite of the confounding measures of cortisol production, rate of appearance of whole body d3-cortisol provides an index of 11 $\beta$ HSD1 reductase activity independently of adrenal production. Again this may be influenced by gender. It has been reported that hepatic and whole body 11 $\beta$ HSD1 activity does not display sexual dimorphism in healthy adults (Finken *et al*, 1999), in keeping with similar whole body d3-cortisol production rates reported here in women and previously in men (Stimson *et al*, 2009). However others have shown enhanced net 11 $\beta$ HSD1 activity in men (Andrew *et al*, 1998; Fraser *et al*, 1999). Urinary glucocorticoid metabolite ratios, an index of whole body 11 $\beta$ HSD1 activity, are also increased in post-menopausal compared to pre-menopausal females (Andersson *et al*, 2009),

but this augmentation reflects an increase in hepatic  $11\beta$ HSD1 rather than subcutaneous adipose tissue  $11\beta$ HSD1 activity. Thus, the peri-menopausal age and hormonal status of the participants is unlikely to have affected tracer-derived measurements of systemic  $11\beta$ HSD1 activity and systemic rate of appearance of d3-cortisol is thought to mainly reflect splanchnic production (Basu *et al*, 2004;Stimson *et al*, 2009).

Other factors may also have impacted on the rates of appearance of cortisol, d3-cortisol and kinetic measurements. For example, the study participants had a wide range of BMIs; subcutaneous adipose tissue  $11\beta$ HSD1 activity is ~3-fold higher (per gram adipose tissue) in obesity (BMI  $31.7\text{kg/m}^2$ ) compared to normal weight (BMI  $22.9\text{kg/m}^2$ ) subjects (Rask *et al*, 2001). However, the numbers in this study were too small to adjust for this statistically.

Another factor that may have influenced the adipose tissue results is the effects of surgery. In skeletal muscle, the physiological stress of abdominal surgery can increase in  $11\beta$ HSD1-reductase activity 5-days post-operatively without changes in transcript levels (Jang *et al*, 2009). However, to my knowledge there are no studies examining  $11\beta$ HSD1 activity both pre-, post- and intra-operatively in adipose tissue. Although anaesthetists were asked to withhold medications known to interfere with glucocorticoid metabolism until the study was complete, all participants received an intravenous infusion of propofol for maintenance of anaesthesia. At plasma concentrations considered necessary for anaesthesia, propofol is unlikely to affect steroidogenesis compared to other agents such as etomidate (Kenyon *et al*, 1985). However, one participant also received pre-operative benzodiazepine, which at high concentrations can inhibit adrenocortical steroidogenesis *in vitro* (Thomson *et al*, 1995), but is not known to modulate activity of  $11\beta$ HSD1.

During a primed continuous infusion of d4-cortisol, steady state is achieved in plasma almost immediately for d4-cortisol itself, within 120 min for d3-cortisone and within 180 min for

d3-cortisol (Andrew *et al*, 2002;Andrew *et al*, 2005). Uptake of steroid into adipose tissue is likely to vary in proportion to the plasma concentration. The net rate of accumulation of d4-cortisol in adipose tissue can therefore be estimated from a single biopsy sample on the assumptions that plasma d4-cortisol concentrations were stable throughout infusion, that d4-cortisol was absent from the adipose tissue before infusion (which was confirmed on archived human subcutaneous adipose samples; data not shown), and that steady state was not achieved in adipose tissue in advance of the biopsy. To confirm the lack of steady state and estimate rates of change, it would have been useful to have multiple biopsies obtained at different time points in each patient, but this was not feasible. However, the disproportionately low concentrations of all deuterated steroids in adipose tissue, compared with those in plasma, suggest that steady state has not been reached in adipose tissue. Unlike d4-cortisol, however, net accumulation rates cannot be estimated so accurately for d3-cortisone and d3-cortisol since plasma concentrations for these steroids will have varied during the early period of infusion as they are generated from d4-cortisol in tissues.

The net rate of accumulation of d4-cortisol into adipose during infusion was estimated at ~0.5 nmol/kg/h (*Equation 6*). This figure reflects the balance between uptake into adipose tissue and any removal, for example by inactivation by enzymes or by export into plasma. The only relevant clearance enzymes expressed in human adipose tissue are 5 $\alpha$ -reductase type 1 (Wake *et al*, 2007b), type 2 (Mackenzie *et al*, 2008) and 11 $\beta$ HSD1, which under some circumstances may catalyse 11 $\beta$ -dehydrogenase activity, converting cortisol to cortisone (Wake *et al*, 2006;White *et al*, 2007). However, a previous study in healthy men undergoing d4-cortisol tracer infusion did not detect removal of d4-cortisol across the subcutaneous or visceral adipose tissue using arteriovenous sampling (Stimson *et al*, 2009), which suggests that the slow rate of accumulation of d4-cortisol in adipose tissue is mainly determined by transport between plasma and intra-adipose steroid pools rather than by rapid enzymatic

inactivation. This contrasts very markedly, for example, with the liver where removal of d4-cortisol from previously published arteriovenous sampling data (Stimson *et al*, 2009) can be calculated as >250nmol/h. It is possible that the slow accumulation reflects relatively low blood flow in adipose tissue (~3ml/ 100g adipose tissue / min (Frayn *et al*, 2003) vs ~400ml/min in liver (Stimson *et al*, 2009)), and/or tissue-specific and steroid-specific differences in active transport of glucocorticoids in and out of tissues (Asif *et al*, 2005;Karssen *et al*, 2001;Lackner *et al*, 1998). Although to my knowledge there are no previous *in vivo* studies assessing cortisol transport and turnover in adipose tissue, one *ex-vivo* study reported adipose tissue uptake of [<sup>3</sup>H]<sub>2</sub>-cortisol was ~5 times slower than that of sex steroids (only 12.7% uptake at 3 hours vs 60.8% and 59.6% for progesterone and testosterone respectively) (Bleau *et al*, 1974).

A key question is whether it can be inferred from slow accumulation of tracer d4-cortisol in adipose tissue that turnover of the endogenous cortisol pool is also slow. Two important issues to address this question are whether there is a difference in d4-cortisol and endogenous cortisol entering the adipose cells, and secondly if 11 $\beta$ HSD1 activity can be detected in adipose, and how much this contributes to the adipose pool.

Firstly, there is no evidence that d4-cortisol and cortisol have different pharmacokinetics or rates of metabolism due to a 'primary isotope effect' by the 11 $\beta$ HSDs (Andrew *et al*, 2002), although this has not been tested with respect to clearance by 5 $\alpha$ -reduction. The affinity of d4-cortisol for transporters has also not been tested. Transporters of steroids into cells have been identified in the adrenal (Asif *et al*, 2005), the central nervous system (Karssen *et al*, 2001), and the liver (Lackner *et al*, 1998), but to my knowledge there have not been any reports of glucocorticoid transporters in adipose tissue. A further consideration is whether transport and binding of d4-cortisol to CBG in the plasma is similar to that of the

endogenous steroid. This has also not been formally tested, but is possible as commercial kits are now available to measure CBG. The absolute concentrations of cortisol in adipose tissue were ~10% those in plasma, consistent with the ~90% binding of cortisol to plasma proteins, making it unavailable for transport into adipose. Concentrations of cortisone were ~20% those of plasma, which is somewhat lower than expected given the low plasma protein binding of cortisone. This might reflect conversion of cortisone to cortisol by 11 $\beta$ HSD1, and the lower cortisone values in subcutaneous adipose tissue may reflect higher enzyme activity in this depot, as suggested in previous studies (Stimson *et al*, 2009).

The principal aim of the study was to assess the rate of formation of d3-cortisol in adipose tissue as an index of 11 $\beta$ HSD1-reductase activity. Theoretically, an established way to achieve this is to calculate the fractional synthetic rate (FSR). To do this, adipose biopsies taken at two different time points are obtained and the change in tracer: tracee ratio (d4-cortisol /d3-cortisol) and rate of appearance of d3-cortisol measured. However, this was not feasible in this study as the adipose biopsies were taken at the beginning of surgery and further biopsies would have interfered with the normal operation. Furthermore, if another biopsy were taken at the end of the operation, this would have prevented anaesthetists administering certain intra-operative drugs such as glucocorticoids for post-anaesthetic nausea and vomiting. Therefore, this approach was not undertaken.

Release of cortisol into plasma from subcutaneous adipose tissue has been demonstrated once before using arteriovenous sampling (Stimson *et al*, 2009). Dilution of d4-cortisol by unlabelled cortisol in adipose tissue can be attributed exclusively to 11 $\beta$ HSD1. The previous study used a similar tracer infusion protocol and carried out arteriovenous sampling in healthy men yielding cortisol production at 9 nmol/kg/h (Stimson *et al*, 2009). Production was not demonstrated across visceral tissues. There are several explanations why additional

dilution of d4-cortisol by d3-cortisol was not detected in adipose tissue compared to plasma in our present study. Firstly, the adipose tissue d4-cortisol pool was not in steady state, and the previous study demonstrating adipose tissue cortisol release did not measure absolute tissue cortisol levels in biopsy samples, simply plasma levels across an arteriovenous gradient (Stimson *et al*, 2009). Secondly, very low concentrations of d3-cortisone were present in adipose tissue, consistent with slow uptake of cortisone as well as cortisol from the plasma, and perhaps providing inadequate substrate for 11 $\beta$ HSD1 activity. Moreover, given that d3-cortisol levels rise rather slowly during d4-cortisol infusion, reaching steady state after up to 3 hours (Andrew *et al*, 2002), one might anticipate that accumulation of d3-cortisol in adipose tissue would lag behind accumulation of d4-cortisol and thus the dilution of d4-cortisol with d3-cortisol would be more marked in plasma than adipose at early time points. The fact that d3-cortisol:d4-cortisol ratios were similar in adipose tissue as in plasma may therefore be an indication of 11 $\beta$ HSD1 activity in adipose tissue, but this cannot be concluded. Indeed, a previous arteriovenous sampling study in men has demonstrated d3-cortisol production in subcutaneous adipose tissue of ~5.2 nmol/kg/h (Stimson *et al*, 2009). This figure may be an underestimate, given that the d4-cortisol infusion lasted only 210 minutes in the previous study and the current data suggest that d3-cortisone substrate concentrations in adipose tissue would still have been low at the time of sampling.

These results suggest that thinking on the short-term metabolic effects of glucocorticoids on adipose tissue needs to be revised. Cortisol accesses intracellular cytosolic glucocorticoid receptors to elicit its effects on fatty acid metabolism and cellular differentiation in adipose tissue. Furthermore, *in vitro* data suggest that ultradian hormone stimulation induces the pulsatile synthesis of RNA from GR-regulated genes (Stavreva *et al*, 2009). It is widely assumed that cortisol gains rapid access to adipose tissue given its lipophilicity, and induces lipolysis in the short term and obesity in the longer term. Certainly, ultradian fluctuations in

plasma glucocorticoids are reflected in hippocampal extracellular fluid in rodents (Droste *et al*, 2008). However, the current data indicating slow turnover between plasma and tissue pools of cortisol are consistent with the intra-adipose cortisol pool integrating the longer-term variations in circulating glucocorticoid concentrations. However, unless there are multiple pools of glucocorticoid within the adipose, with a cytosolic ‘free’ pool that turns over more quickly than a triglyceride-bound pool, then it appears unlikely that intra-adipose cortisol concentrations vary widely during acute changes in plasma cortisol. This suggestion is supported by the suggestion that ~90% of an adipocyte is composed of lipid, whilst only ~10% is composed of an aqueous phase (personal communication from Dr C Keynon). Therefore, we speculate that the majority of the glucocorticoid pool in adipose tissue is unlikely to be affected by acute changes in plasma glucocorticoids. Thus, diurnal variation in intra-adipose cortisol concentrations is likely to be small, but this delayed rate needs to be confirmed under normal physiological circumstances, for example where adipose blood flow, meal pattern and insulin levels normally occur, rather than during the acute physical stress of surgery. Moreover, the rates of regeneration of cortisol by intra-adipose 11 $\beta$ HSD1 measured in previous studies are high compared with the rate of accumulation of plasma-derived cortisol within adipose tissue, emphasising the importance of 11 $\beta$ HSD1 in human adipose tissue glucocorticoid signalling. Differences in glucocorticoid responses between visceral and subcutaneous adipose tissue are not explained by differences in uptake of cortisol. It will be important to establish the rates of exchange between plasma and other intra-cellular cortisol pools in other organs.

## **Chapter 4**

### **Quantifying cortisone production / $11\beta$ - dehydrogenase activity in man using deuterated cortisone**



This chapter addresses aim 4 from Chapter 1 Introduction, to develop a stable isotope tracer method with 1,2- $[^2\text{H}]_2$ -cortisone to measure cortisone production, reflecting 11 $\beta$ -dehydrogenase activity *in vivo*.

## 4.1 Introduction

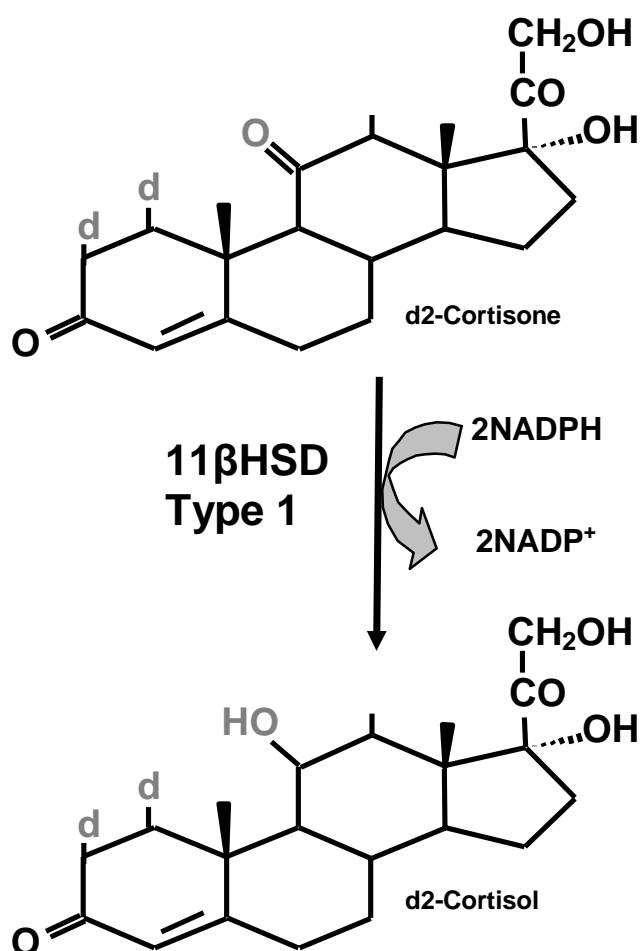
Cortisone is an inert steroid that is produced endogenously exclusively upon 11 $\beta$ -dehydrogenation of cortisol. The 11 $\beta$ -dehydrogenation reaction, converting cortisol to cortisone in man, is catalysed by 11 $\beta$ HSD2, eg in the distal nephron (Stewart & Krozowski, 1999). As described in section 1.6.1, 11 $\beta$ HSD2 functions to rapidly inactivate cortisol and reserve MR specifically for aldosterone.

Conversely, 11 $\beta$ HSD type 1, a low affinity NADP-dependent microsomal enzyme, is expressed in many tissues and functions predominantly as a reductase, regenerating cortisol from cortisone (Seckl & Walker, 2001). Selective inhibitors of 11 $\beta$ HSD1 are in development for treating type 2 diabetes by lowering tissue cortisol levels (Hughes *et al*, 2008). However, recent data suggest that, in the absence of NADPH cofactor generation by hexose-6-phosphate dehydrogenase, 11 $\beta$ HSD1 can catalyse predominant dehydrogenase inactivation of cortisol (Lavery *et al*, 2006). Furthermore, 11 $\beta$ HSD1-dehydrogenase activity has been reported in ASV primary cell culture experiments and in *in vivo* subcutaneous adipose tissue microdialysis studies (Bujalska *et al*, 2002; Wake *et al*, 2006), but has not been quantified *in vivo*. Measurement of cortisone production by 11 $\beta$ -dehydrogenase *in vivo* will be key to determining whether these observations are important in physiological regulation of tissue cortisol, the consequences of pathological dysregulation, and the effects of novel enzyme inhibitors.

Current methods for measuring 11 $\beta$ -dehydrogenase activity and/or cortisone production *in vivo* are inadequate. Most investigators have relied upon measuring urinary free cortisol:cortisone ratios as an index of renal 11 $\beta$ HSD2 activity (Best & Walker, 1997;Palermo et al, 1996), or urinary cortisol:cortisone metabolite ratios which reflect predominantly intra-hepatic steroid levels. However, these ratios only reflect net balance between the activities of multiple enzymes and do not quantify the rates of turnover between cortisol and cortisone. Other investigators have administered labelled substrates for 11 $\beta$ -dehydrogenase, including 11 $\alpha$ -[<sup>3</sup>H]-cortisol (Hellman et al, 1971), 9,11,12,12-[<sup>2</sup>H]<sub>4</sub>-cortisol (Andrew et al, 2002), 11 $\alpha$ -[<sup>2</sup>H]-cortisol (Kasuya *et al*, 2005), and 1,2-[<sup>2</sup>H]<sub>2</sub>-cortisol (Vierhapper *et al*, 2007) but the rate of removal of some of these compounds are not exclusively dependent on 11 $\beta$ -dehydrogenase, and rates of accumulation of 11 $\beta$ -dehydrogenase product (labelled cortisone, or liberated [<sup>3</sup>H]) cannot be quantified accurately in steady state in the absence of simultaneous measurement of clearance of the product. No investigators have yet undertaken the gold standard approach in which endogenous cortisone production is inferred from dilution of ‘tracer’ labelled cortisone, infused in steady state. For ethical reasons surrounding ionizing radiation, and because of the specificity of simultaneous analysis of tracer and tracee by mass spectrometry in the presence of their metabolites, stable isotope tracers are preferred to radioisotopes for this purpose.

The stable isotope tracer 1,2-[<sup>2</sup>H]<sub>2</sub>-cortisone (d2-cortisone) was chosen as the compound to develop a method to study 11 $\beta$ -dehydrogenase *in vivo*. d2-Cortisone consists of a cortisone molecule with 2 deuteriums attached to the 1,2 positions of the steroid A-ring. When infused systemically we hypothesised that d2-cortisone would be reduced by 11 $\beta$ -reductase to form d2-cortisol, with both deuteriums remaining on the product (Figure 4.1). This was proposed as the deuteriums on d2-cortisone are not placed on carbon 6 or 11 $\alpha$  of the steroid molecule,

the main sites where hydrogen atoms are lost during hydroxylation (by CYP3A4) or oxidation by 11 $\beta$ HSDs respectively.



**Figure 4.1: Proposed metabolism of d2-cortisone by 11 $\beta$ HSD type 1.**

This tracer could then be infused *in vivo* to assess cortisone production systemically (net production by 11 $\beta$ HSD1 and 11 $\beta$ HSD2) or used to measure arteriovenous differences in cortisone production across tissue beds. Changes in cortisone production may then be quantified in specific pathways or under pharmaceutical manipulation.

## 4.2 Hypothesis and Aim

The hypothesis was that 11 $\beta$ HSD1 catalyses dehydrogenation of cortisol *in vivo* generating cortisone. The aim was to develop and validate a novel stable isotope tracer, d2-cortisone, to quantify net whole body cortisone production rate, and hence 11 $\beta$ -dehydrogenase activity *in vivo*.

## 4.3 Methods

### 4.3.1 Assay Development

#### *Optimising measurement of d2-cortisone using LC-MS/MS*

d2-Cortisone and d2-cortisol (1mg/ml) were dissolved in mobile phase and infused into the source of the LC-MS/MS (for further details of the instrument see section 2.8.2). The protonated molecular ions were subjected to a range of tube lens voltages and temperatures to ascertain optimum ionisation conditions. LC-FT-MS was undertaken to identify the exact mass of the ions formed and determine their molecular formulae.

#### *Interferences*

Specificity of detection of d2-cortisone in the presence of endogenous steroids was assessed, to ensure the substance detected was d2-cortisone and not other related naturally occurring mass isotopomers of cortisone, for example molecules with carbon 13 (natural abundance of  $^{13}\text{C}$  1.11%) and deuteriums (natural abundance of  $^2\text{H}$  0.015%). The following interferences were quantified: cortisone (mass+2,  $m/z$  363), which may interfere with d2-cortisone

analysis, and cortisol (mass+2  $m/z$  365) which may interfere with d2-cortisol analysis. However, the exact composition of the isotopomer detected e.g either  $^{13}\text{C}$  or  $^2\text{H}$  could not be determined. The purity ie absence of unlabelled steroid of both d2-cortisol and d2-cortisone was also assessed.

To further characterise purity of d2-cortisone, d2-cortisone (10 $\mu\text{g}$ ) was derivatised to form methoxime-trimethylsilyl derivatives (section 2.7.3) and analysed using a full scan protocol by GC-MS system as per section 2.8.4.

### ***Assay validation***

#### *Linearity of quantitation*

Increasing amounts of cortisone and d2-cortisone were injected onto the column to calibrate quantitation. Epi-cortisol was used as an internal standard, by addition of 500ng to each sample. The calibration curve was plotted by calculating the peak area ratio of analyte versus that of internal standard (epi-cortisol, y axis) against the amount of added analyte (x axis) and a line of best fit was drawn in the form  $y=mx+c$  (please see Figure 4.5).

#### *Ionisation efficiency*

Equivalence of ionisation of tracer and tracee (ie d2-cortisone and cortisone) was assessed using an enrichment curve. The enrichment is the sum of the tracer divided by the tracee plus tracer (enrichment =  $\text{tracer} / (\text{tracer} + \text{tracee})$ ). To assess the linearity of this relationship with instrumental response an enrichment curve with fixed proportions of tracer to tracee was prepared.

### *Quality control*

Accuracy and precision of analysis of d2-cortisone was assessed at the lowest (2.5ng) and highest (200ng) concentrations of the standard curves, and weighting adjusted to maximise the RME (accuracy) and RSD (precision) (de Bievre *et al*, 1998). The limit of detection (LOD) and limit of quantification (LOQ) were also determined (section 2.8.2).

### **4.3.2 *In vitro* studies**

#### ***In vitro assessment of whether d2-cortisone is a substrate for 11 $\beta$ HSD1-reductase***

To establish that the experimental tracer, d2-cortisone, was a substrate for reduction by human 11 $\beta$ HSD1 and to compare the reaction kinetic parameters between the two substrates, d2-cortisone or cortisone (2 $\mu$ M) were incubated with HEK293h11 $\beta$ HSD1 cells (2x10<sup>5</sup>) (Webster *et al*, 2007) (n=3) under conditions described in section 2.5.3. Steroids were extracted as described in section 2.6.2 and the presence of d2-cortisol and cortisol respectively assessed by LC-MS/MS (section 2.8.2).

#### ***Assessment of a primary isotope effect of deuterium on the velocity of metabolism of substrate by 11 $\beta$ HSD1-reductase***

The stable isotope tracer contains 2 deuterium atoms in place of 2 hydrogen atoms in endogenous cortisone, which may influence the strength of interactions with the enzyme active site. To assess if this affected the kinetics of 11 $\beta$ HSD1, the velocities of product formation (d2-cortisol vs cortisol) were compared from the above experiment (n=3).

To test for a primary isotope effect using a competition approach; [ $^3\text{H}$ ] $_2$ -cortisone (5nM) was incubated with increasing concentrations of cortisone or d2-cortisone (0nM - 4955nM) with HEK293h11 $\beta$ HSD1cells ( $2 \times 10^5$ ) (n=3), under conditions described in section 2.5.3. Steroids were extracted as per section 2.6.2, and the velocity of formation of [ $^3\text{H}$ ] $_2$ -cortisol quantified following analysis by HPLC with radio-detection (section 2.8.1)

***Assessment of a primary isotope effect on the velocity of metabolism of substrate by 5 $\beta$ -reductase /3 $\alpha$ -hydroxysteroid dehydrogenase***

As 5 $\beta$ -reduced glucocorticoids are metabolised quickly by 3 $\alpha$ HSDs *in vitro* (Iyer *et al*, 1990), we were unable to assess metabolism of d2-cortisone by these two enzymes individually. Therefore human hepatic cytosol was used to assess the combined action of these two enzymes resulting in formation of 3 $\alpha$ ,5 $\beta$ -tetrahydrocortisone or its d2-labelled isotopomer.

For assay development, liver tissue from a lean Zucker rat, aged 12 weeks was used. The liver was removed from the animal intact, snap frozen on dry ice and stored at -80°C until required. Archived human liver tissue was kindly donated by Dr Roger Brown, from an anonymised stored tissue sample obtained 1994/95. Initial informed consent obtained in 1994/5 allowed use of the tissue for research in general. Advice confirming that the tissue was suitable for use under the ethical permission given and the Human Tissue (Scotland) Act 2006 was obtained from Dr Rachel Smith, Training & Communications Co-ordinator, Medical Research Council (MRC) Regulatory Support Centre, Edinburgh.

To test for a primary isotope effect, cytosol (778 $\mu\text{g/ml}$  protein) (n=3) were incubated with cortisone or d2-cortisone substrate (10 $\mu\text{M}$ ) and [ $^3\text{H}$ ] $_2$ -cortisone (10nM) under conditions

described in section 2.4.1. Steroids were extracted as per section 2.6.1 and the velocity of the product quantified following analysis by HPLC with radio-scintillation detection (section 2.8.1). Cold steroidal standards were subsequently co-infused with the tritiated reaction product to identify the steroids formed. The reaction was also repeated with cold substrate only and the products derivatised (section 2.7.3) and analysed by GC-MS (section 2.8.4) to confirm the identity of the product.

#### **4.3.3 *In vivo* clinical studies investigating d2-cortisone metabolism**

All *in vivo* studies were approved by the Local Research Ethics Committee and all subjects gave written informed consent.

##### ***In vivo pharmacokinetic study: using a single bolus injection***

###### ***Subjects***

To assess pharmacokinetic characteristics of d2-cortisone, healthy lean male volunteers (n=3) on no regular medication, who had not received glucocorticoid treatment by any route for 3 months, with normal biochemical indices (haemoglobin, renal, thyroid, liver function tests and glucose), attended the Clinical research Facility at 08:30h after an overnight fast.

###### ***Preparation of the stable isotope tracer d2-cortisone***

d2-Cortisone (500µg) was dissolved in 1.0ml pharmaceutical grade ethanol and filtered in the radio pharmacy to form a sterile stock solution. For a single bolus dose, 0.3ml of stock solution (containing 150µg of d2-cortisone) was added to sodium chloride 0.9% w/v (49.7ml)



to make a total volume of 50ml (3 $\mu$ g/ml). 3.0ml of the diluted solution was discarded to leave 47.0ml (containing 141 $\mu$ g of d2-cortisone).

#### *Clinical measurements*

Blood pressure was measured after sitting for at least 10 minutes using a 705IT automatic blood pressure monitor (OMRON Healthcare Europe BV, Hoofddorp, NL) prior to the start of the study.

#### *Clinical administration protocol*

One cannula was placed using an aseptic technique into each antecubital fossa vein. Venous blood was collected for basal assessment of steroids and background isotopomers. An intravenous bolus of d2-cortisone was administered (141 $\mu$ g in 0.9% w/v saline, 50ml) over 5 minutes into the contra-lateral arm. Venous blood was obtained at frequent intervals from 0 to 90 minutes from the contra-lateral arm to the bolus injection.

#### *Sample collection and processing*

Prior to obtaining any blood samples, 2ml of dead space (a mixture of saline and blood in the cannula) was withdrawn from the sampling cannula to prevent contamination of the whole blood sample by saline. Samples for plasma enrichment with d2-cortisone were obtained in 5.5ml lithium heparin S-Monovette tubes. Blood samples were placed on wet ice prior to centrifugation (1000 x g 10min, 4°C) to obtain plasma which was harvested and stored at -20°C.

### *Sample analysis*

Endogenous and deuterated glucocorticoids were extracted from plasma as described in section 2.6.3 and analysed by LC-MS/MS (section 2.8.2).

### *Measuring cortisone production rate in vivo by steady state infusion*

#### *Subjects*

To measure net endogenous cortisone production rate at steady state, male volunteers, (n=3) attended the Clinical Research Facility at 08:30h after an overnight fast.

#### *Preparation of the stable isotope tracer d2-cortisone*

d2-Cortisone (500µg) was dissolved in 1.0ml ethanol and filtered in the radio pharmacy to form a stock solution. From the stock, 0.2ml (containing 100µg d2-cortisone) was added to sodium chloride 0.9% v/w (49.8ml) to make a total volume of 50ml (3µg/ml). 12.0ml of the diluted solution was discarded to leave 38.0ml (containing 76µg d2-cortisone) to be administered as a loading dose. For the infusion, 0.8ml (containing 400µg of d2-cortisone) of stock solution was added to sodium chloride 0.9% v/w (499.2ml).

#### *Clinical measurements*

Blood pressure was measured after sitting for at least 10 minutes using a 705IT automatic blood pressure monitor (OMRON Healthcare Europe BV, Hoofddorp, NL) prior to the start of the study.

### *Clinical protocol*

A priming dose (76µg in 0.9% saline) was administered followed by a 3-hour infusion of d2-cortisone (105µg/hour, 0.9% saline). Venous blood was sampled at intervals from the contralateral arm over 3 hours. Spot morning urine and saliva samples (~8:30am) (one sample of each) were obtained before the infusion.

### *Sample collection and processing*

Blood samples were collected, processed and stored as per section 4.3.3. Urine samples were collected in a plain universal container and saliva samples were obtained by asking the volunteer to chew on cotton wool sticks (Salivette, Sarstedt, Beaumont Leys, UK) for 1 minute. Salivettes underwent centrifugation (3000 x g 10min, 4°C) and urine and saliva were stored at -20°C. A sample of d2-cortisone infusate was collected at the end of the study and stored at -20° C.

### *Sample analysis*

Endogenous and deuterated glucocorticoids were extracted from plasma as per section 2.6.3 and analysed by LC-MS/MS (section 2.8.2). Urine and salivary glucocorticoids were extracted as per sections 2.6.5 and 2.6.4 and analysed by GC-MS and LC-MS/MS respectively (sections 2.8.4 and 2.8.2).

### ***Effect of inhibition of 11 $\beta$ -dehydrogenase by liquorice on cortisone production rate***

To test the effect of inhibition of 11 $\beta$ -dehydrogenase with liquorice on cortisone production rate, subjects in the ‘measuring cortisone production rate *in vivo* by steady state infusion’ study attended a second time after eating 200g of black liquorice (Panda liquorice chews 3.8% liquorice extract, Vaajakoski, Finland) per day for 2 days. The study protocol above was repeated.

#### **4.3.4 Calculations and data analysis**

Quantitation of steroids was performed as described in section 2.8.2. Tracer: tracee ratios (TTRs) of peak areas of d2-cortisol:cortisol and d2-cortisone:cortisone were corrected for the abundances of naturally occurring isotopomers at baseline. Concentrations of d2-cortisone and endogenous cortisol and cortisone in plasma were determined using calibration curves as described in section 2.6.3. As a standard for d2-cortisol was not available initially, plasma d2-cortisol concentrations were calculated by multiplying total cortisol concentration by the TTR d2-cortisol:cortisol. As the d2-cortisone tracer contained ~10-15% cortisone impurity, plasma cortisone concentrations were corrected using *Equation 1*.

*Equation 1:*

Corrected plasma [cortisone] (nM) =

Plasma [cortisone] (nM) - (% cortisone in tracer powder • plasma [d2-cortisone] (nM))

### *Kinetic analysis*

Volume of distribution, half-life and clearance of d2-cortisone were calculated from data obtained following bolus injection using Kinetica software (Thermo Scientific, Philadelphia, PA), by fitting the data to a one compartmental model.

To calculate the amount of d2-cortisone to administer as a bolus dose and achieve a plasma concentration of 30nM, *Equation 2* was used.

*Equation 2:*

$$\text{Bolus dose } (\mu\text{g}) = \text{Concentration required in plasma } (C_p) \cdot \text{Volume of distribution } (V_d)$$

To confirm the amount of d2-cortisone to administer as a bolus dose and continuous infusion to achieve steady state plasma concentrations of ~10nM, *Equations 2 and 3* were used.

*Equation 3:*

$$\text{Infusion rate} = C_{ss} \text{ (nM)} \cdot \text{Clearance (L.min}^{-1}\text{)}$$

To calculate clearance for *Equation 3*, *Equation 4* was used, where  $K_{el}$  is the elimination constant (mins):

*Equation 4:*

$$\text{Clearance} = V_d \cdot K_{el}$$

Clearance was calculated at steady state plasma data (ss, t=60-180mins) using *Equation 5*.

*Equation 5:*

Clearance ( $\text{L} \cdot \text{min}^{-1}$ ) = rate of infusion ( $\text{nmol} \cdot \text{min}^{-1}$ ) / Steady state concentration ( $C_{ss}$ , (nM))

Net rate of appearance ( $R_a$ ) of cortisone in the plasma was calculated from the mean TTR (d2-cortisone:cortisone) in steady state (*Equation 6*). Rate of d2-cortisone infusion was derived from analysis of study infusates.

*Equation 6:*

Net  $R_a$  Cortisone = rate of d2-cortisone infusion (nmol/min)/ TTR (d2-cortisone:cortisone)

### *Statistical analyses*

Data are presented as mean  $\pm$  SEM and were compared using paired Student's t-tests.

## **4.4 Results**

### **4.4.1 Mass spectrometric analysis**

Ionisation parameters, mass transitions and structural composition of ions monitored during LC-MS/MS are summarised in Table 4.1. The proposed fragmentation mechanism of d2-cortisol and d2-cortisone within the mass spectrometer is displayed in Figure 4.2. The protonated molecular ions of the d2-cortisone and d2-cortisol differed from cortisone and

cortisol by 2 mass units respectively, whereas d2-cortisol formed a product ion differing by only one mass unit from cortisol, due to loss of deuterium following fragmentation across the steroidal B ring, confirmed by FT-MS. Fragmentation of d2-cortisol also resulted in a product ion with  $m/z$  ratio of 329 at low collision energies (13V) so the sum of the abundances of these two ions was used for quantitative analysis.

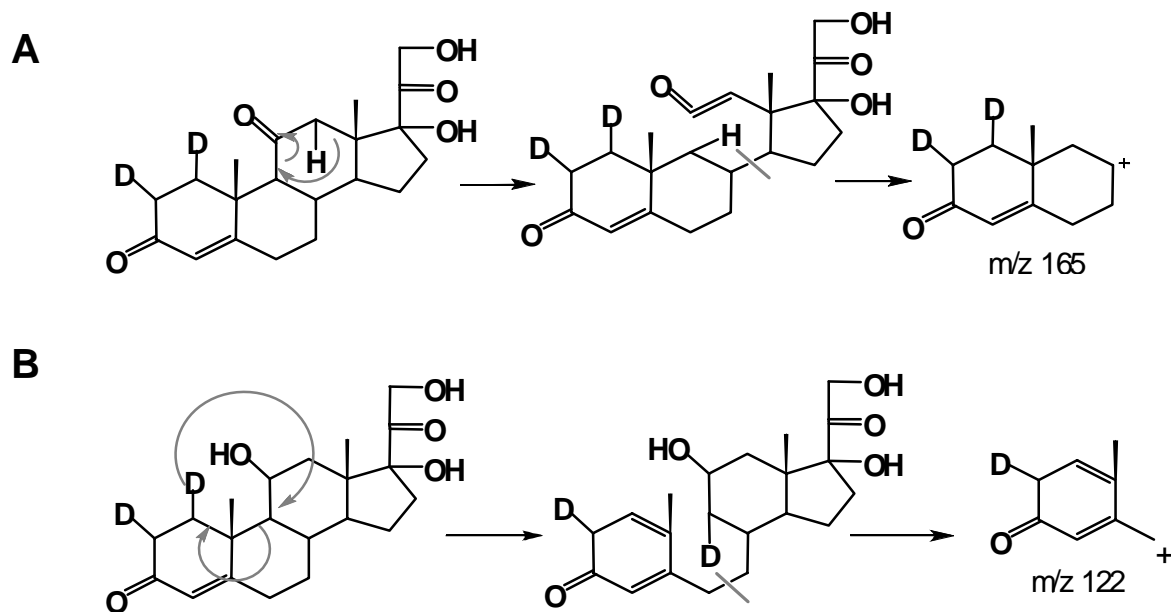
Cortisone was ionized with greater efficiency than d2-cortisone and this was taken into account by correcting values obtained in experimental samples using the enrichment curve (Figure 4.3).

**Table 4.1: Chemical formulae, mass transitions and MS ionisation conditions for endogenous and deuterated steroids**

Compound	Parent ion <i>m/z</i>	Product ion <i>m/z</i>	Collision energy (V)	Tube lens (V)	Product accurate mass (amu)	*Δppm	Empirical formula
Cortisone	361	163	22	168	163.1114	-2.2546	C <sub>11</sub> H <sub>15</sub> O
Cortisol	363	121	31	142	121.0647	-0.4000	C <sub>8</sub> H <sub>9</sub> O
d2-Cortisone	363	165	21	118	165.1239	-2.397	C <sub>11</sub> H <sub>13</sub> D <sub>2</sub> O
d2-Cortisol	365	122	23	118	122.0710	-0.5659	C <sub>8</sub> H <sub>8</sub> D <sub>1</sub> O
d2-Cortisol	365	329	13	118	329.2079	0.4572	C <sub>21</sub> H <sub>25</sub> D <sub>2</sub> O <sub>3</sub>

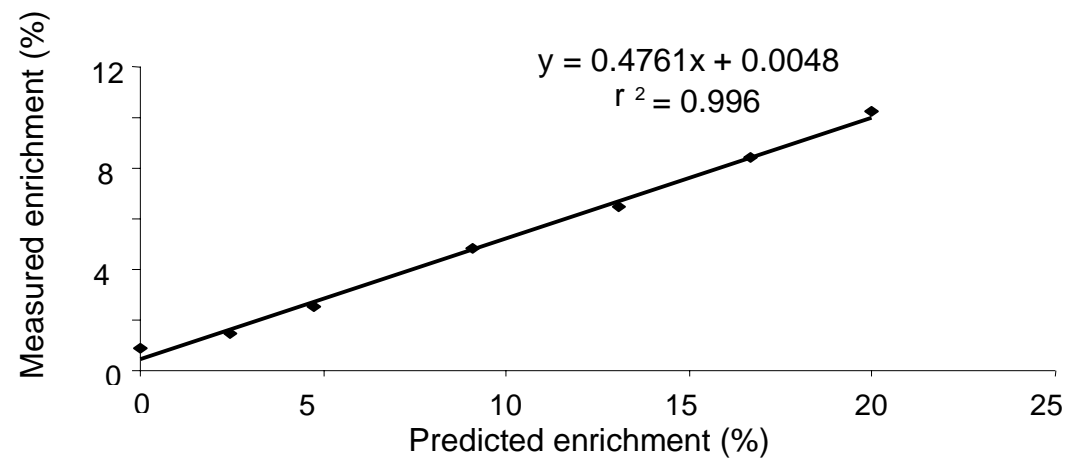
V=Volts, Δppm = delta parts per million from calculated accurate mass, \*assessed by the LTQ Orbitrap FT-MS, amu= atomic mass units, d= deuterium, D= deuterium under empirical formula; *m/z*= mass charge ratio.





**Figure 4.2: Proposed fragmentation mechanisms for (A) d2-cortisone and (B) d2-cortisol during mass spectrometric analysis.**

A) d2-Cortisone fragmentation across the steroidal C-ring resulting in a product ion with 2 deuteriums attached and  $m/z$  165. B) d2-Cortisol fragmentation across the steroidal B-ring results in a protonated product ion with  $m/z$  122 retaining 1 deuterium atom. D= deuterium,  $m/z$  = mass charge ratio. Curly arrow indicates a one electron transfer; + = a positively charged ion.



**Figure 4.3: Enrichment curve for tracer (d2-cortisone) and tracee (cortisone)**

Enrichment (Enrichment = tracer / tracer + tracee). To assess the equivalence of ionisation an enrichment curve with fixed proportions of tracer (d2-cortisone) to tracee (cortisone) was prepared. From the curve, it is apparent that the ratio of tracer to tracee was not 1:1, eg a fixed enrichment of 20% reads as a value of 10% for d2-cortisone / cortisone. Thus the enrichment curve was used to correct data when calculating cortisone concentrations *in vivo*.

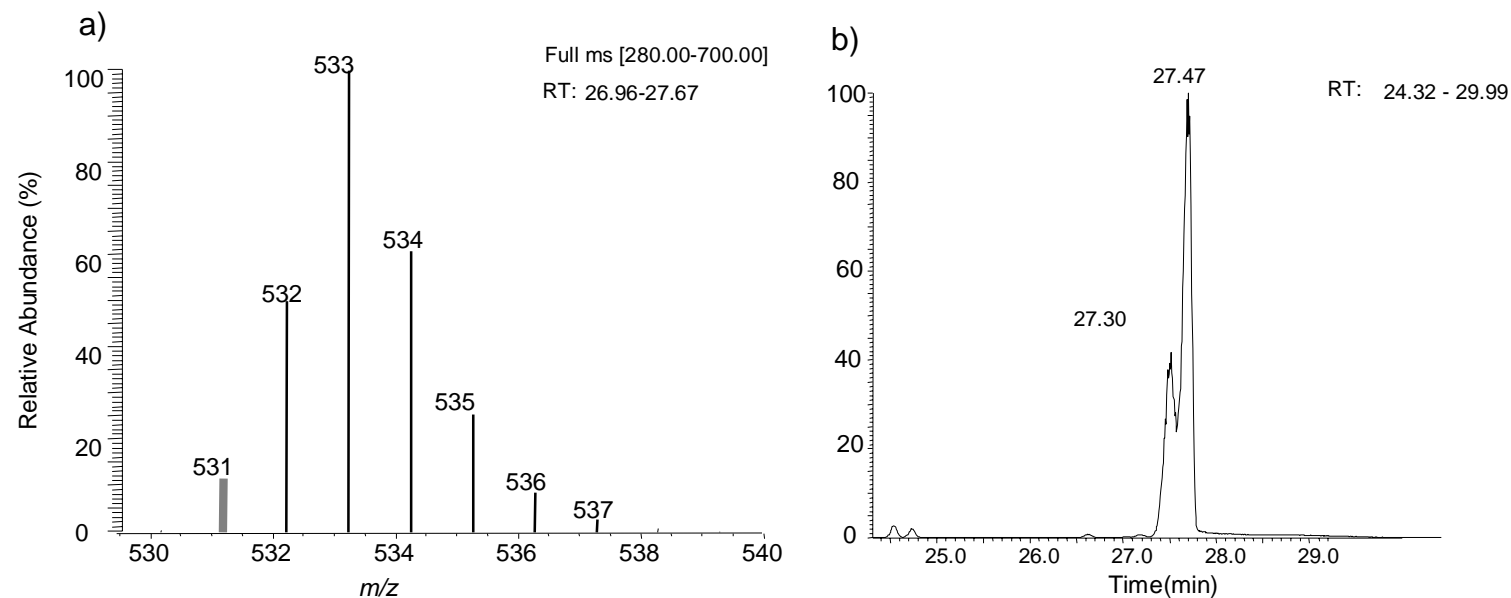
## ***Interferences***

Using selective reaction monitoring (SRM), endogenous cortisone (mass+2,  $m/z$  363) gave a signal equivalent to 1% of the cortisone peak area which would interfere with d2-cortisone. It was determined that this background isotopic interference did not contribute significantly to the d2-cortisone signal as in plasma it did not exceed noise, and therefore did not need to be corrected for when assessing d2-cortisone concentrations, although this can be readily achieved mathematically.

However, a ~15% interference of the d2-cortisone peak area was detected when infusing d2-cortisone at an ion with a  $m/z$  of 361 (i.e. equivalent to cortisone). Possible explanations for this interference included inaccurate isotopic-labelling during manufacture of the tracer, contamination of d2-cortisone during sample preparation, or detection of a different naturally occurring contaminant with  $m/z$  361 in the LC-MS.

To identify the cause of the  $m/z$  361 interference, fresh stock steroid solutions were prepared and analysed on a full scan protocol of a Finnigan Voyager GC-MS (ie a different analytical instrument). A sample with reagents only was analysed as a control and the interference was not detected. Approximately 15% contamination remained when the derivatised d2-cortisone standard was analysed ( $m/z$  ion of methoxime-trimethylsilyl derivative of cortisone =  $m/z$  531 (Andrew *et al*, 2002)) suggesting that the isotopic enrichment of the d2-cortisone tracer was less than 98% deuterated as claimed by the manufacturer manufacture (Figure 4. 4). This was subsequently confirmed on analysis of a different batch of tracer. The manufacturer was unable to provide a purer powder. Thus the contribution of this contamination to the signal for endogenous cortisone was corrected for mathematically by subtracting the amount of endogenous cortisone measured in a sample of d2-cortisone powder (dissolved in methanol) from the concentration of cortisone in experimental samples.

**Figure 4.4 GC-MS full scan of methoxime-trimethylsilyl (MO-TMS) derivative of d2-cortisone**



To identify the cause of the  $m/z$  361 interference, a solution of d2-cortisone was derivatised to its MO-TMS derivatives and analysed on a full scan protocol using a Finnigan Voyager GC-MS. (a) Approximately 15% contamination remained (thick grey bar) when the derivatised d2-cortisone (MO-TMS  $m/z$  533) standard was analysed ( $m/z$  ion of methoxime-trimethylsilyl derivative of cortisone =  $m/z$  531). The compound with  $m/z$  532 may represent an m-1 isotopomer of d2-cortisone. (b) Mass chromatogram of isomers of MO-TMS derivative of d2-cortisone.  $m/z$  = mass charge ratio; RT= retention time.

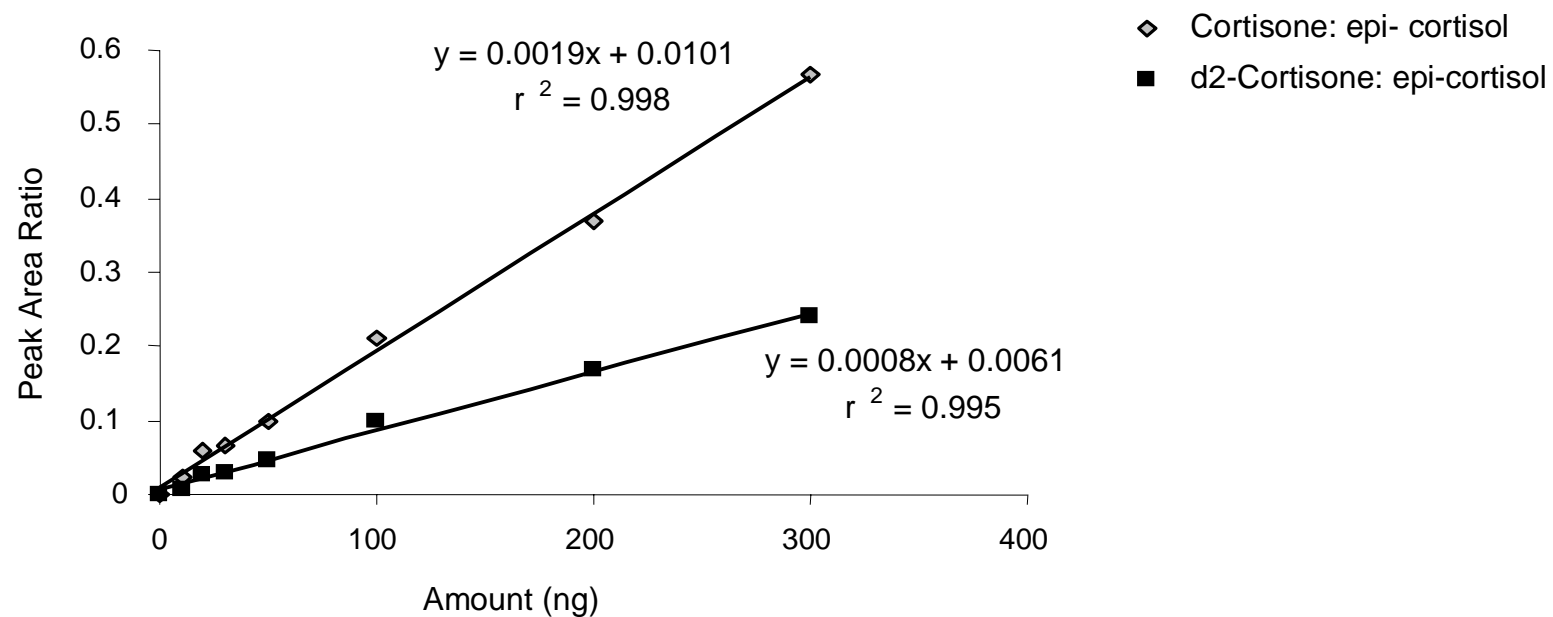
The presence of naturally occurring mass isotopomers was also assessed in solutions of both d2-cortisol and cortisol. Using SRM, cortisol (mass+2,  $m/z$  365) gave a signal equivalent to 3% of the cortisol peak area in d2-cortisol. In contrast to the d2-cortisone standard which contained a significant amount of cortisone, the d2-cortisol standard did not contain any measurable cortisol. The presence of mass isotopomers detected was not significantly greater than background and did not need correction.

### ***Assay validation***

All the responses of analytes exhibited a linear relationship with concentration, when quantified using an internal standard; regression lines of calibration curves had  $r^2 > 0.99$ . This relationship was linear over a wide range of amounts of physiological relevance (Figure 4.5)

Despite injecting equal amounts of cortisone and d2-cortisone, the peak area ratios of d2-cortisone were smaller than those of cortisone, yielding a calibration line with a less steep gradient, suggesting that the deuterated cortisone was not ionised with the same efficiency as the non-deuterated steroid in the LC-MS. Thus data pertaining to d2-cortisone was corrected using an enrichment curve. Similar inequalities were encountered with d2-cortisol since two transitions were monitored for d2-cortisol, but only one for cortisol.

The limit of detection (LOD) for the d2-cortisone assay was determined as 1ng, with a signal to noise ratio  $>3$ . The limit of quantification (LOQ) was determined as 2.5ng at which intra-assay RSD and RME were 7.0% and -0.1% respectively. Inter-assay RSD and RME were 7.5% and 2.3% respectively. At the highest point on the standard curve (200ng) intra-assay RSD and RME were 5.7% and 2.3% and inter-assay RSD and RME 9.1% and -2.8% respectively. Applying a weighting of  $1/\chi^2$  to the d2-cortisone calibration line improved RME.



**Figure 4.5: Cortisone and d2-cortisone calibration curves with epi-cortisol internal standard.**

Increasing amounts of cortisone (grey diamonds) and d2-cortisone (black squares) were analysed by LC-MS/MS to calibrate quantitation. Epi-cortisol (500ng) was used as an internal standard. The calibration curve was plotted by calculating the peak area ratio of analyte versus that of internal standard (epi-cortisol, y axis) against the amount of added analyte (x axis).

#### 4.4.2 *In vitro* metabolism of d2-cortisone

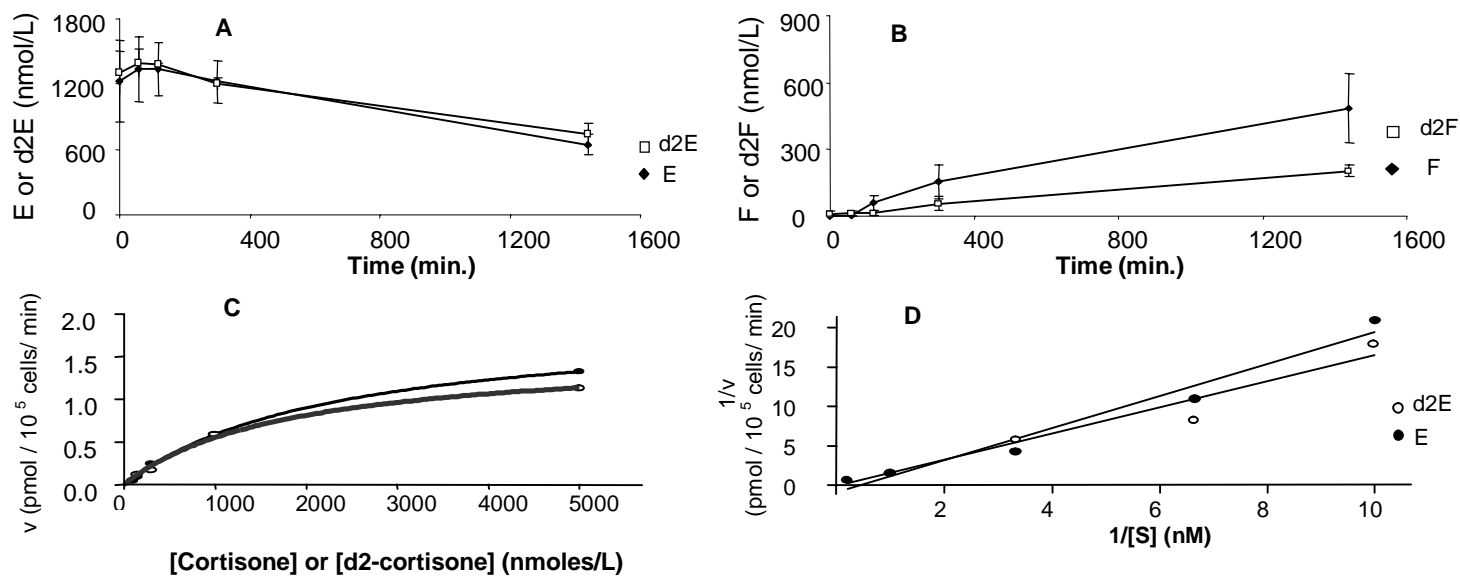
##### *d2-Cortisone is a substrate for human 11 $\beta$ HSD1-reductase*

d2-Cortisone was a substrate for human 11 $\beta$ HSD1 in that incubation of HEK293h11 $\beta$ HSD1 cells with d2-cortisone or cortisone resulted in production of d2-cortisol or cortisol, respectively, at similar rates ( $0.38 \pm 0.20$  vs  $1.05 \pm 0.50$  pmol/ $10^5$  cells/min,  $p=0.19$ ). d2-Cortisone and cortisone concentrations declined at a similar rate (Figure 4.6A) and d2-cortisol and cortisol concentration increased (Figure 4.6B). Similarly, d2-cortisone and cortisone had comparable abilities to compete with [ $^3\text{H}$ ]<sub>2</sub>-cortisone for metabolism:  $V_{\max}$  for formation of [ $^3\text{H}$ ]<sub>2</sub>-cortisol in the presence of d2-cortisone was  $1.80 \pm 0.64$  and in the presence of cortisone was  $2.18 \pm 0.78$  pmol/ $10^5$  cells/min ( $p=0.56$ ); apparent  $K_m$  was  $2.29 \pm 1.05$  vs  $2.76 \pm 1.23 \mu\text{M}$  ( $p=0.57$ ), respectively (Figure 4.6 C and D). In control samples without cells or without [ $^3\text{H}$ ]<sub>2</sub>-steroid there was no evidence of [ $^3\text{H}$ ]<sub>2</sub>-cortisone reduction to [ $^3\text{H}$ ]<sub>2</sub>-cortisol.

##### *d2-Cortisone is a substrate for human 5 $\beta$ -reductase/3 $\alpha$ -hydroxysteroid dehydrogenase*

In hepatic cytosol, 5 $\beta$ -reduced cortisol metabolites are rapidly reduced by 3 $\alpha$ HSDs *in vitro*, resulting in only the tetrahydro-steroids being measurable (Iyer *et al*, 1990). Human hepatic cytosols converted [ $^3\text{H}$ ]<sub>2</sub>-cortisone to [ $^3\text{H}$ ]<sub>2</sub>-3 $\alpha$ ,5 $\beta$ THE. The identity of the product was confirmed using HPLC with UV detection (Figure 2.1) and a assay with cold substrate only and the product confirmed by GC-MS. Cortisone and d2-cortisone were metabolised at similar rates by 5 $\beta$ -reductase/3 $\alpha$ HSD ( $0.050 \pm 0.004$  vs  $0.045 \pm 0.006$  pmol/mg/h,  $p=0.71$ ). A further, as yet unidentified, peak formed from ~15% of substrate levels was obtained (velocity:  $0.069 \pm 0.002$  vs  $0.084 \pm 0.008$  pmol/mg/h,  $p=0.15$ ). [ $^3\text{H}$ ]<sub>2</sub>-Products were not formed in control samples without cytosol or co-factor.

**Figure 4.6: *In vitro* metabolism of d2-cortisone and cortisone by HEK 293 cells stably transfected with human 11 $\beta$ HSD1**



A) d2-Cortisone (d2E) or cortisone (E) were extracted from media of HEK293/h11 $\beta$ HSD1) cells during incubation with d2E or E, respectively. B) d2-Cortisol (d2F) or cortisol (F) were extracted from media of HEK293/h11 $\beta$ HSD1 cells incubated with d2E or E, respectively. C) Michaelis-Menten and D) Lineweaver-Burke plots demonstrating the velocity of the reaction with HEK293/h11 $\beta$ HSD1 cells incubated with increasing concentrations of E or d2E and a fixed quantity of tritiated cortisone ( $[^3\text{H}]_2\text{E}$ ).  $1/\text{S}$  = reciprocal of substrate concentration,  $1/\text{V}$ = reciprocal of velocity. All data are mean  $\pm$  SEM for n=3 in duplicate. Differences in kinetic data generated during d2E and E incubation were not statistically significant, compared by Student's t test.



#### 4.4.3 *In vivo* kinetics

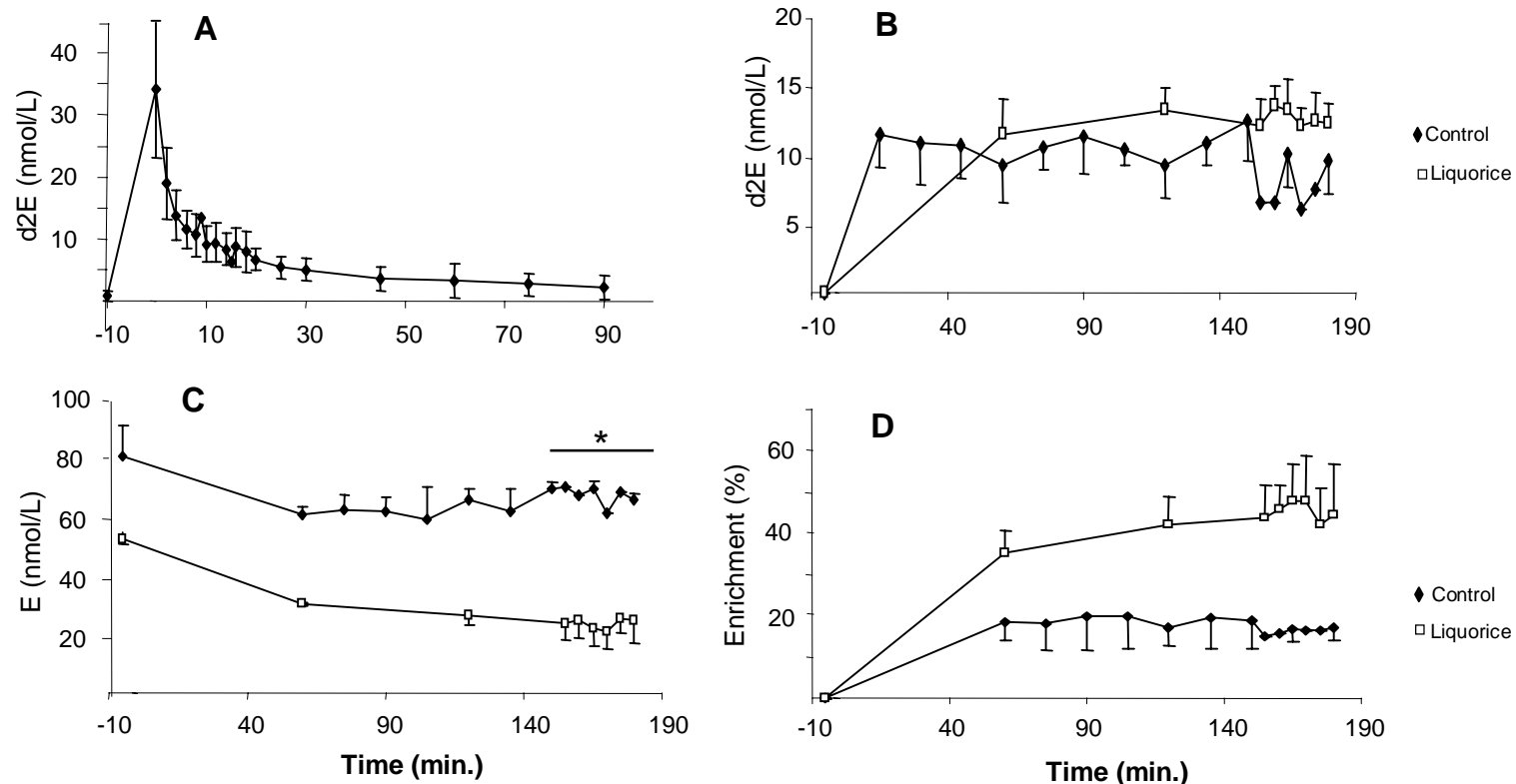
To assess the pharmacokinetic behaviour of d2-cortisone, a bolus dose of d2-cortisone was administered to 3 healthy male volunteers. The mean age of participants was 36 (range 29-49) y, and mean BMI 23.0 (18.8-26.5) kg /m<sup>2</sup>. After bolus administration, elimination of d2-cortisone from blood conformed to first order kinetics and the tracer could be detected for 1.5h after injection (Figure 4.7A). The half life was 57.5 min, volume of distribution 47.0 L, and area under the curve 494nmol/L/min. This data was used to inform the administration protocol for the steady state infusion aiming to achieve steady state plasma concentrations of 10nM.

To assess cortisone production rates, the tracer was infused at a rate of 105.3µg/h into participants with characteristics detailed in Table 4.2. d2-Cortisone was not detected in plasma at baseline. During infusion of d2-cortisone for 3 hours, cortisone, cortisol, d2-cortisone and d2-cortisol were readily measured in plasma. Steady state d2-cortisone concentrations of 9.3±2.1nmol/L were achieved after just 15 minutes (Figure 4.7B), with mean plasma cortisone levels at steady state of 64.9±2.6nmol/L (Table 4.2). The dilution of d2-cortisone by endogenous cortisone resulted in a calculated net rate of appearance of cortisone of 40.4 ± 10.0 nmol/min.

To assess if the technique for measuring dilution of d2-cortisone was sensitive enough to detect changes in cortisone production rates, liquorice was administered to the same healthy volunteers. Following liquorice, endogenous cortisone levels were significantly lower (Figure 4.7C). There was a trend towards a rise in enrichment of plasma cortisone with d2-cortisone (Figure 4.7D), which resulted in a ~50% reduction in the calculated net rate of appearance of cortisone (p=0.05) (Table 4.2). Plasma cortisol:cortisone ratios in a pre-infusion sample increased (Table 4.2), however, neither urinary ratios (cortisol:cortisone or

cortisol:cortisone metabolite ratios) nor salivary cortisone concentrations were significantly affected by liquorice (Table 4.2). Cortisol was below the limit of quantitation in saliva.

**Figure 4.7: *In vivo* d2-cortisone administration**



Healthy volunteers were studied before (♦) and after (◻) administration of liquorice. A) Plasma d2-cortisone (d2E) concentrations after an intravenous bolus of d2E. B) Plasma d2E concentrations following infusion of tracer with and without liquorice administration. C) Plasma cortisol (E) concentrations following infusion of tracer were lower after taking liquorice. D) Plasma enrichment of E with d2E following infusion of tracer. All data are mean  $\pm$  SEM for n=3. \* = p<0.05 comparing plasma steady state concentrations before and after liquorice using paired Student's t-tests.

**Table 4.2: Participant characteristics and kinetic parameters of cortisolone and its tracer, d2-cortisone.**

	Without liquorice n=3	With liquorice n=3	P value
Age (y)	35.3±6.8	-	-
BMI (kg/m <sup>2</sup> )	22.8±2.1	23.6±2.3	0.29
SBP (mmHg)	123±6	133±6	0.36
DBP (mmHg)	86±5	84±2	0.51
<b>Before tracer infusion</b>			
Plasma cortisol (nmol/L) ~9am	287.8±46.3	339.8±24.4	0.03
Plasma cortisolone (nmol/L)	80.7±10.5	52.5±1.9	0.14
Plasma cortisol:cortisone	3.6±0.3	6.5±0.5	0.04
Urine cortisol:cortisone	3.0±0.2	3.9±1.1	0.42
Urine cortisol:cortisone metabolites ((5βTHF+5αTHF)/THE)	1.2±0.1	1.4±0.2	0.58
Salivary cortisolone (nmol/L)	8.2±3.7	4.9±1.0	0.54
<b>During tracer infusion</b>			
C <sub>ss</sub> cortisolone (nmol/L)	64.9±2.6	24.8±4.7	0.02
C <sub>ss</sub> d2-cortisone (nmol/L)	9.3±2.1	12.6±1.7	0.25
Enrichment d2-cortisone (%)	16.9±2.9	43.5 ±8.0	0.08
Rate of appearance of cortisone (nmol/min)	40.4±10.0	21.7±2.9	0.05
Clearance d2-cortisone (L/min)	1.04±0.8	0.9±0.2	0.83
C <sub>ss</sub> cortisol (nmol/L)	238.0 ±33.7	177.9 ±40.5	0.18

All data are mean ± SEM for n=3. BMI= Body Mass Index, SBP= Systolic Blood Pressure, mmHg = millimetres of Mercury, DBP= Diastolic Blood Pressure, C<sub>ss</sub>= Concentration at steady state, d= deuterium, THF= tetrahydro-cortisol, THE = tetrahydro-cortisone.

## 4.5 Discussion

Using the above *in vitro* and *in vivo* studies a new stable isotope tracer method has been developed and validated to quantify cortisone production by 11 $\beta$ -dehydrogenase activity *in vivo* in humans. Tracer measurement was optimised using LCMS/MS, and the data demonstrate that d2-cortisone is metabolised in a similar fashion to cortisone *in vitro* not only by human 11 $\beta$ HSD1, but also by other cortisone metabolising enzymes in liver, indicating that there is no significant primary isotope effect from the deuteriums and the metabolism of the tracer is similar to the tracee. Moreover, using liquorice, the rate of appearance of cortisone is more sensitive as an indicator of inhibition of 11 $\beta$ -dehydrogenase activity than the existing methods of urinary cortisol:cortisone ratio or salivary cortisone analysis.

Mass spectrometric analysis was used to confirm mass transitions of d2-cortisone, its metabolite, d2-cortisol, and the structural composition of the ions monitored during LC/MS-MS. The pattern of d2-cortisone fragmentation was similar to endogenous cortisone with the exception that the 2 'tracer' deuteriums remained on the product fragment. d2-Cortisol fragmentation differed to cortisol as a deuterium atom remained on the product after tandem mass spectrometry, however specific mass transitions could be assigned to distinguish tracer and tracee. Naturally occurring isotopomers of cortisone did not contribute significantly to the d2-cortisone signal and the LC-MS/MS assay was validated demonstrating suitable limits of quantification and detection for assays.

In order for a compound to be a suitable tracer for *in vivo* analysis the following assumptions must hold true. Firstly, the tracer must be a substrate for the enzyme and metabolised and cleared at the same rate as the endogenous compound. Utilising HEK293h11 $\beta$ HSD1 cells, it

was apparent that d2-cortisone was a substrate for 11 $\beta$ HSD1 and was metabolised to d2-cortisol at a similar rate to that of endogenous cortisone to cortisol. Furthermore, by assessing the combined actions of 5 $\beta$ -reductase and 3 $\alpha$ -HSD, it was demonstrated that d2-cortisone and cortisone are metabolised at a similar rate resulting in the production of 5 $\beta$ ,3 $\alpha$ -tetrahydrocortisone or its d2-isotopomer. However, other routes of glucocorticoid metabolism were not assessed such as the 20 $\alpha$ - or 20 $\beta$ -hydroxysteroid dehydrogenases or cytochrome P450s, but these do not make a major contribution to total rates of clearance, at least in health, unlike A-ring reduction.

A second assumption is that the tracer must not be converted into the tracee. In the case of d2-cortisone, stripping of the deuteriums from the tracer would result in the production of a molecule identical to that of endogenous cortisone (ie the tracee) and add to the tracee pool if infused into humans. As the deuteriums on d2-cortisone are not positioned on carbon 6 or 11 $\alpha$  (sites where loss might occur through metabolism), it was hypothesised that this should not occur. To test this hypothesis, the mass transitions of d2-cortisone and d2-cortisol were monitored from extracts of *in vitro* cell studies. During, LC-MS/MS analysis, no obvious loss of all deuteriums occurred. However, the issue of whether stripping of deuteriums occurs *in vivo* has not been fully proven as endogenous cortisone (ie the product of stripping deuteriums from d2-cortisone) is present in human plasma samples. To try and assess if deuteriums are lost from the tracer *in vivo*, d2-cortisone could be administered to a rodent which has corticosterone and 11-dehydrocorticosterone as its endogenous glucocorticoids. Analysis of the rodent plasma should reveal endogenous glucocorticoids and d2-cortisone and d2-cortisol. If any unlabelled cortisone is detected it would indicate stripping of deuteriums *in vivo*.

Thirdly, when developing a stable isotope tracer method, one must ensure that tracer recycling does not occur or is accounted for. Some glucose tracers such as 3-[<sup>13</sup>C]-glucose

do recycle *in vivo* and this enables calculation of the rate of glucose cycling (Wolfe & Chinkes, 2005). However, if the isotope is taken up by a tissue, metabolised, and re-appears in the system as the tracer again because of recycling, the calculated rate of appearance will be under-estimated by that amount (Wolfe & Chinkes, 2005). d2-Cortisone may initially be reduced to d2-cortisol and then dehydrogenated back to d2-cortisone by the 11 $\beta$ HSDs. Indeed, this recycling occurs *in vivo* with endogenous cortisone. To address this issue, in a further set of healthy volunteers (n=6, please see Chapter 5), d2-cortisone and d4-cortisol were co-infused as part of a separate study. The rate of d2-cortisone to d2-cortisol conversion was calculated from the Ra d3-cortisol assuming this to be the same as the rate of d2-cortisol conversion to d2-cortisone and that inter-conversion remains to be in linear proportions to the substrate concentration (please see p178). As d4-cortisol is not regenerated from d3-cortisol, recycling by 11 $\beta$ HSD1-reductase does not need to be accounted for with this tracer. The rate of recycling of d3-cortisone to d3-cortisol was estimated as ~8nmol/min, which would increase the rate of appearance of cortisone in this study by ~15-20%.

Having established the suitability and possible limitations of using d2-cortisone to trace cortisone, a series of experiments were performed to assess endogenous cortisone production. To design an infusion protocol, pharmacokinetics of the tracer were established initially. The results from the bolus injection of d2-cortisone suggest that d2-cortisone has a longer half-life than has been previously reported for cortisone (57.5 vs. 28min) (Peterson *et al*, 1957). However, the earlier report from the 1950s relied on older assay technology assessing the counter-current distribution of [<sup>14</sup>C]-labelled steroids reacting with phenylhydrazine and the report does not comment whether a primary isotope effect was assessed or indeed exists with [<sup>14</sup>C]-cortisone.

Cortisone production rate was also somewhat higher than the rate previously calculated using non-steady state kinetics with a d4-cortisol tracer (40.4 vs. 24.1nmol/min, adjusted for

substrate concentration and  $V_d$  (Andrew *et al*, 2002)), but this likely reflects the advantages of making steady state measurements based on dilution of tracer by tracee, when both tracer and tracee are at physiological concentrations, rather than extrapolation of initial rates of metabolism. Notably, the cortisone production rate calculated here is approximately 60-70% of the cortisol production rate calculated in similar healthy men using d4-cortisol infusion (Andrew *et al*, 2002), confirming that  $11\beta$ -dehydrogenase is a major route of cortisol metabolism.

After short-term liquorice administration plasma endogenous cortisone concentrations fell substantially, consistent with impaired  $11\beta$ -dehydrogenase and accounting for the rise in plasma cortisol:cortisone ratio. Differences in ratios of urinary free cortisol:cortisone and cortisol:cortisone metabolites were not statistically significant, but were in the expected directions. Previous investigators demonstrating significant changes in urinary metabolites used longer periods of liquorice treatment (Stewart *et al*, 1987). Thus urinary indices may change significantly in the longer term, but the approach using d2-cortisone tracer is able to pick up changes in  $11\beta$ -dehydrogenase activity in the shorter term.

The primary source of cortisone production is likely to be the kidney (Hellman *et al*, 1971; Whitworth *et al*, 1989) from dehydrogenation of cortisol, but further studies using d2-cortisone tracer will be able to dissect this further eg by arteriovenous sampling across tissue beds. Alternative sites of cortisone production include the gut (Hirasawa *et al*, 1997) or blood vessels (Christy *et al*, 2003). This study and the majority of published work estimating cortisone production (Hellman *et al*, 1971; Andrew *et al*, 2002; Vierhapper *et al*, 2007) have used intra-venous infusions of isotopes which should reduce / exclude any contribution from the gut. Theoretically another potential source of cortisone in man could be the reversible oxidation of tetrahydrocortisone or even tetrahydrocortisol (via tetrahydrocortisone by



11 $\beta$ HSD2). This is highly unlikely as although hepatic 3 $\alpha$ -HSDs can act reversibly to form dihydrocortisone, reversibility of 5 $\beta$ -reductase has not been demonstrated (Bush & Mahesh, 1959).

The major advantage of d2-cortisone steady state tracer infusion over existing techniques is that it is specific to the 11 $\beta$ -dehydrogenase activity: if 11 $\beta$ -reductase activity is altered this will affect tracer and tracee equally. Other tools based on the ratio of cortisol:cortisone or of their metabolites reflect only the net balance of dehydrogenase and reductase activities, and are influenced by changes in either. By achieving steady state within 15 minutes, d2-cortisone infusion will be relatively quick and easy to incorporate into investigation protocols. In principle, d2-cortisone can also be combined with d4-cortisol infusion (Andrew *et al*, 2002) to measure 11 $\beta$ -dehydrogenase and 11 $\beta$ -reductase activities simultaneously. Disadvantages include the need for mass spectrometry, but this is required for urinary steroid ratios too, and the care that must be taken to take account of background isotopomer enrichments.

Dissection of 11 $\beta$ HSD dehydrogenase from reductase becomes important in tissues where both may exist either due to both 11 $\beta$ HSD1 and 11 $\beta$ HSD2 being present or through reversibility of 11 $\beta$ HSD1. In states of NADPH deficiency eg when 11 $\beta$ HSD1 is liberated from the intracellular environment or when H6PDH is lost through gene targeting in mice (Lavery *et al*, 2006), 11 $\beta$ HSD1 displays dehydrogenase activity. Adipose tissue 11 $\beta$ HSD1 also demonstrates some dehydrogenase activity in primary cell cultures and in intra-adipose microdialysis *in vivo* (Bujalska *et al*, 2002; Wake *et al*, 2006). As human adipose tissue 11 $\beta$ HSD2 expression is negligible and 11 $\beta$ HSD1 expression is abundant (Bujalska *et al*, 2002), any dehydrogenase activity in human adipose tissue can be attributed to 11 $\beta$ HSD1.

In conclusion, d2-cortisone tracer can now be used to measure cortisone generation in whole body and in specific tissues to further our understanding of the role of 11 $\beta$ HSDs in physiological control of tissue steroid levels, pathological amplification of cortisol concentrations in hypertension and obesity, and pharmacological targeting. In the next chapter of this thesis, Chapter 5, d2-cortisone is used to measure 11 $\beta$ -dehydrogenase activity in subcutaneous adipose tissue and forearm skeletal muscle *in vivo* to explore in more detail the equilibrium of 11 $\beta$ HSD1 in these tissues.

## **Chapter 5**

### **Inter-conversion of cortisol and cortisone in human subcutaneous adipose tissue and skeletal muscle *in vivo***

This chapter addresses aims 5 and 6 in Chapter 1 Introduction, applying the newly developed tracer method for d2-cortisone and measuring 11 $\beta$ -dehydrogenase activity in the whole body, across subcutaneous adipose tissue, forearm skeletal muscle and assessing the effect of increased insulin and glucose flux on 11 $\beta$ HSD1 activity and directionality.

## 5.1 Introduction

Cortisol is generated locally in tissues through the conversion of inert cortisone to cortisol by the intracellular enzyme 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (Seckl & Walker, 2001). Recent studies using a stable isotope tracer 9,11,12,12-[<sup>2</sup>H]<sub>4</sub>-cortisol show that *in vivo* adipose tissue 11 $\beta$ HSD1 predominantly functions as a reductase generating 15pmol of cortisol per min/100g tissue (Stimson *et al*, 2009). 11 $\beta$ HSD1-reductase activity is driven by NADPH co-factor derived from hexose-6-phosphate dehydrogenase (H6PDH), co-localised with 11 $\beta$ HSD1 in the endoplasmic reticulum lumen (White *et al*, 2007). Altered cortisol generation in tissues such as adipose tissue and the liver has been implicated in the metabolic complications of obesity (Rask *et al*, 2001), and 11 $\beta$ HSD1 inhibition has been investigated as a potentially useful therapeutic target to lower intracellular cortisol levels (Hughes *et al*, 2008). However, in states of NADPH deficiency eg when 11 $\beta$ HSD1 is liberated from the intracellular environment or when H6PDH is lost through gene targeting in mice (Lavery *et al*, 2006), 11 $\beta$ HSD1-dehydrogenase activity predominates. It has been demonstrated that adipose tissue 11 $\beta$ HSD1 has some dehydrogenase activity, inactivating cortisol to cortisone, in primary cell cultures and in intra-adipose microdialysis *in vivo* (Bujalska *et al*, 2002; Wake *et al*, 2006). As human adipose tissue 11 $\beta$ HSD2 expression is negligible and 11 $\beta$ HSD1 expression is abundant (Bujalska *et al*, 2002), any dehydrogenase activity in human adipose tissue can be attributed to 11 $\beta$ HSD1 and this may be metabolically protective (Kershaw *et al*, 2005).

11 $\beta$ HSD1 is also expressed in skeletal muscle (Jang *et al*, 2006), an important site of insulin resistance in type 2 diabetes. In rodents skeletal muscle 11 $\beta$ HSD1 activity is low compared to adipose (Livingstone *et al*, 2000), whilst in humans the evidence for *in vivo* skeletal muscle 11 $\beta$ HSD1 activity is equivocal as no gradient in cortisol and cortisone concentrations was found in forearm muscle (Katz *et al*, 1999) but a small amount of reductase activity was detected in the leg using the stable isotope tracer 9,11,12,12-[<sup>2</sup>H]<sub>4</sub>-cortisol (Basu *et al*, 2004; Basu *et al*, 2005). *Ex-vivo* studies have demonstrated skeletal muscle 11 $\beta$ HSD1-reductase activity and a small amount of 11 $\beta$ HSD1-dehydrogenase activity in tissue homogenates (Jang *et al*, 2007). Since increased 11 $\beta$ HSD1 expression in skeletal muscle associates with diabetes (Jang *et al*, 2007; Morgan *et al*, 2009) it is important to clarify its function.

To quantify 11 $\beta$ HSD1 dehydrogenase activity in adipose tissue and skeletal muscle *in vivo*, a novel stable isotope tracer 1,2-[<sup>2</sup>H]<sub>2</sub>-cortisone has been developed and validated (see Chapter 4). Using 1,2-[<sup>2</sup>H]<sub>2</sub>-cortisone in combination with 9,11,12,12-[<sup>2</sup>H]<sub>4</sub>-cortisol (Andrew *et al*, 2002) simultaneous 11 $\beta$ HSD1-reductase and dehydrogenase activity can be measured in humans *in vivo*.

## 5.2 Aim

To use the isotopes 1,2-[<sup>2</sup>H]<sub>2</sub>-cortisone (d2-cortisone) and 9,11,12,12-[<sup>2</sup>H]<sub>4</sub>-cortisol (d4-cortisol) in conjunction with arteriovenous sampling techniques to selectively measure 11 $\beta$ HSD1 activity in adipose tissue and skeletal muscle of healthy subjects. Secondly to test the effect of hyperinsulinaemia on whole body, subcutaneous adipose tissue and forearm skeletal muscle 11 $\beta$ HSD1 activity and directionality; hypothesising that increasing glucose uptake may increase substrate supply to H6PDH and drive a switch from dehydrogenase to reductase activity (Wake *et al*, 2006).

## **5.3 Methods**

### **5.3.1 Study Design**

The study was approved by the Local Research Ethics Committee (Milton Keynes Research Ethics Committee) and all subjects gave written informed consent.

#### ***Subjects***

Six healthy male individuals were studied. The volunteers were recruited from the Oxford Clinical Research Bio-bank during 2009. The inclusion criteria included healthy non-obese men aged 20-70, with no glucocorticoid use within the last 3 months, alcohol intake <28 units/ week and normal screening blood tests (full blood count, urea and electrolytes, thyroid and hepatic function and glucose). Study participants were aged between 30-49 years (mean  $\pm$ SEM 42.3 $\pm$ 3.6y), with a body mass index of 21.1-27.6kg/m<sup>2</sup> (24.6 $\pm$ 0.9) and fat mass of 10.5-30.5kg (16.5 $\pm$ 2.9).

#### ***Clinical measurements***

Height and clothed weight were measured using standard techniques as described in Chapter 3, section 3.3. Dual energy X-ray absorptiometry (DXA) (Lunar, GE Healthcare, Chalfont St Giles, UK) scanning was undertaken to assess total fat and lean body mass. Prior to inclusion in the study ultrasound screening of the vessels to be cannulated was undertaken by clinical staff from the Clinical Research Unit, Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, Oxford.

### ***Preparation of the study infusions***

#### *d4-Cortisol (40% d4-cortisol and 60% hydrocortisone)*

The stock solution was made by dissolving 12mg of cortisol and 8mg d4-cortisol (total 20mg) in 2.0ml ethanol in a sterile airflow hood in the Oxford Clinical Research Facility. For the bolus injection, 0.4ml (containing 4mg total cortisol) of stock solution was dissolved in sodium chloride 0.9% v/w (19.6ml) (total volume 20mls). 2.5ml of this solution was discarded and the remaining 17.5ml (containing 3.5mg of total cortisol) injected over 5 minutes. For the infusion, 1.2ml of stock solution (containing 12mg of total cortisol) was dissolved in sodium chloride 0.9% v/w (248.8ml) to make a total volume of 250mls (0.048mg / 1ml). This was infused at a rate of 36.3ml (1.74mg of total cortisol) per hour.

#### *d2-Cortisone*

To make the stock solution, 1.0mg of d2-cortisone was dissolved in 2.0ml ethanol (500µg/ml). For the loading dose, 0.2ml (containing 100µg of d2-cortisone) of stock solution was added to sodium chloride 0.9% (9.8ml, total volume 10mls). 2.4ml of the solution was discarded and the remaining 7.6ml (containing 76µg of d2-cortisone) infused over 5 minutes. For the continuous infusion, 1.4ml of stock solution (containing 700µg of d2-cortisone) was mixed with sodium chloride 0.9% (248.6ml) to achieve a total volume of 250ml (2.8µg d2-cortisone / 1ml). This solution was infused at 37.6ml/h (105.3µg/h).

### *Actrapid insulin*

Actrapid (0.2ml, 200 units) was added to sodium chloride 0.9% (57ml) and 2.3ml of the subjects blood, to give a final concentration of 350 milliunits/ ml, and infused according to the study protocol.

### *Clinical protocol*

To measure forearm skeletal muscle and subcutaneous adipose tissue 11 $\beta$ HSD1 reductase and dehydrogenase activity, volunteers attended the Clinical Research Facility between 07.15-07.30am after an overnight fast to a quiet temperature controlled room. With the volunteer supine, under ultrasonic guidance, a Careflow catheter (150mm, 20-gauge, BD, Oxford, UK) was placed into a superficial vein on the anterior abdominal wall with the tip just superior to the inguinal ligament. Catheter placement allowed access to the venous drainage of the subcutaneous adipose depot, with only a minor contribution from the skin (Frayn *et al*, 1989). To ensure blood collected was from the adipose depot and not from abdominal muscle, the O<sub>2</sub> saturation of the blood was confirmed >85%. One retrograde cannula (20-gauge) was then placed under ultrasonic guidance into a deep branch of the median cubital vein in the left antecubital fossa to obtain access to venous drainage from the forearm skeletal muscle with only minor contributions from the skin and adipose tissue of the forearm (Butler & Home, 1987) and O<sub>2</sub> saturation confirmed as <40%. A further retrograde cannula in a vein draining the right hand, was warmed to 60°C in a hot-box to arterialise the blood sampled (Roddie *et al*, 1956) and O<sub>2</sub> saturation confirmed >98%. A fourth cannula (18-gauge) was placed antegradely into the right antecubital fossa for the study infusions. The cannulae were kept patent by continuous slow infusion of 0.9% saline (w/v, 30ml/h).



At t=-10 minutes, an arterialised blood sample was collected for assessment of basal endogenous glucocorticoid levels and background isotopomers and an intravenous bolus of d2-cortisone (76.0µg, 0.9% saline, w/v) and d4-cortisol (40:60 d4-cortisol: cortisol, 3.5mg, 0.9% saline, w/v) administered into the antegrade antecubital fossa cannula over 5 minutes (Figure 5.1). Continuous intravenous infusions of d2-cortisone (105.3 µg /hour) and d4-cortisol (1.74mg/hour) (both in 0.9% saline w/v) were commenced immediately thereafter. Arterialised samples were obtained at 60 minute intervals to track enrichment of the blood with the tracers.

From t=+3h, 4 sets of blood samples were obtained simultaneously from the 3 sites, (abdominal, forearm and arterialised cannulae), at 10 minute intervals. Prior to taking samples from the deep forearm vein a wrist cuff was inflated to 200mmHg for 2 minutes to remove blood flow from the hand. Forearm blood flow was measured by intermittent occlusion of the upper arm with a cuff inflated to 40mmHg during the sampling period using a strain gauge and a forearm venous occlusion plethysmography unit (Hokanson, Bellevue, WA). Adipose tissue blood flow was measured continuously during the study using Mediscint gamma-counter probes after a subcutaneous injection of 1-2 MBq <sup>133</sup>Xenon lateral to the umbilicus (Larsen *et al*, 1966). This tracer washout technique relies on measuring the initial slope of the <sup>133</sup>Xenon washout curve which is proportional to adipose tissue blood flow. Flow can be calculated from the plot of disappearance of <sup>133</sup>Xenon counts versus time using the following equation, where the partition co-efficient refers to the partition between the lipid and aqueous phase of adipose and has a ratio of 10:1 with xenon gas.

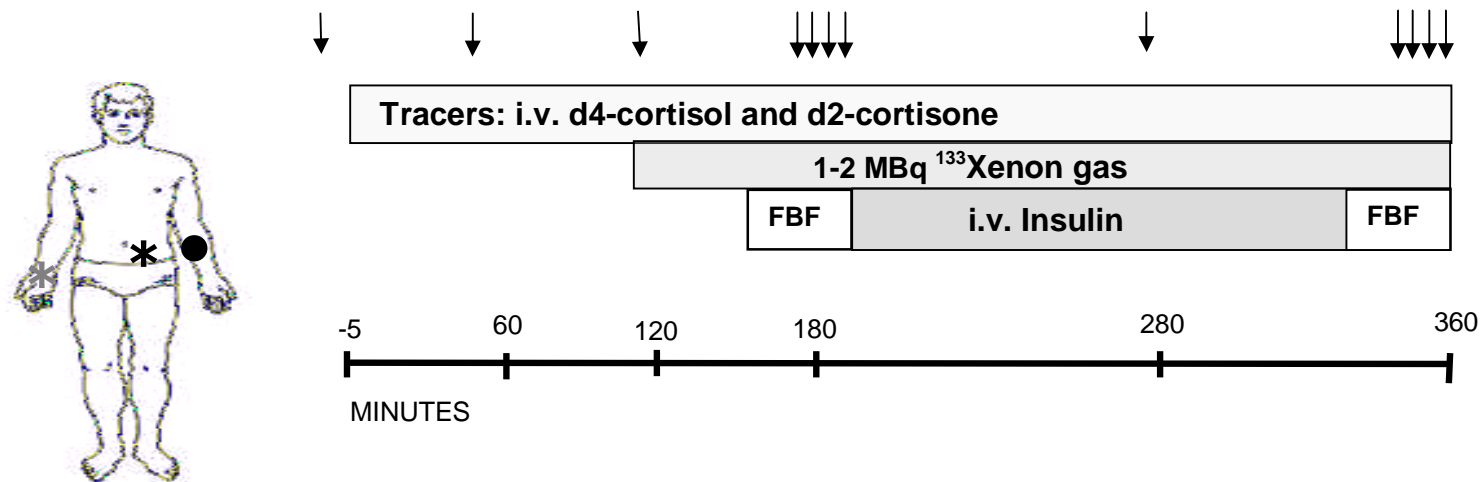
Adipose tissue blood flow (ml/ min/100g tissue) =

Slope of the plot of disappearance of counts • (60sec • 100g tissue• 10 partition co-efficient).

After the sampling period, a hyperinsulinaemic-euglycaemic clamp was commenced by administering a primed continuous infusion of soluble Actrapid insulin with a final infusion rate of  $35\text{mU/m}^2/\text{min}^{-1}$ . To prevent adhesion of the insulin to plastics in the syringe, 2.3ml of the participant's blood was added to the solution (DeFronzo *et al*, 1979). To maintain an arterialed blood glucose concentration of  $\sim 5\text{mmol/L}$ , 20% dextrose (w/v) was co-administered at a variable rate and blood glucose measured at 5-minute intervals using a reflectometer (Hemocue B-glucose analyser, Sheffield, UK). Estimates of the amount of dextrose to infuse during the study for each participant were calculated from a figure in the original description of the hyperinsulinaemic clamp protocol (DeFronzo *et al*, 1979) and the dextrose infusion rate changed by 5-10ml every 5 minutes until plasma glucose levels were stable. After 2h of hyperinsulinaemia, 4 sets of blood samples were obtained simultaneously from the 3 sites at 10 minute intervals and adipose and forearm blood flow was measured.

### ***Sample collection and processing***

Prior to obtaining any blood samples, the pumps infusing saline to keep cannulae patent were switched off for  $\sim 30$  seconds and then 2ml of dead space / waste (a mixture of saline and blood in the cannula) was withdrawn from the sampling cannula. Prior to taking samples from the deep forearm vein a wrist cuff was inflated to 200mmHg for 2 minutes to reduce venous contribution from the hand. Whole blood samples were obtained in lithium heparin S-Monovette tubes (Starstedt, Beaumont Leys, UK). Blood samples were placed on wet ice prior to centrifugation ( $1900 \times g$  10min,  $4^\circ\text{C}$ ) to obtain plasma and stored at  $-20^\circ\text{C}$ .



**Figure 5.1 Arterio-venous sampling study protocol.**

The tracers, d4-cortisol and d2-cortisone, were infused for 360 minutes after placement of cannulae. Intravenous insulin and dextrose was commenced at t=+210 mins. Arrows indicate blood sampling; FBF = forearm blood flow measured by venous occlusion plethysmography; \* = sampling sites- grey arterialised, black superficial epigastric vein, • = deep forearm vein; MBq= mega-Becquerel; i.v = intravenous.

### 5.3.2 Analytical techniques

#### *Plasma*

Endogenous and deuterated glucocorticoids were extracted from plasma using the method detailed in section 2.6.3. Extracts were analysed using LC-MS/MS as per section 2.8.2. The LC-MS/MS assay was optimised to assess specificity of detection of d2-cortisone and d4-cortisol and their metabolites and ensure that there were no interfering isotopomers when the tracers were co-infused. The following interferences were quantified: d2-cortisone (mass +1,  $m/z$  364), which would interfere with d3-cortisone analysis; d2-cortisol (mass +1,  $m/z$  366) which would interfere with d3-cortisol analysis; and d2-cortisol (mass +2,  $m/z$  367) would interfere with d4-cortisol analysis if both d4-cortisol and d2-cortisone were simultaneously infused.

Plasma glucose was measured within 24h on fresh plasma to avoid the variable loss of glucose on freezing.

#### *Whole blood*

Whole blood oxygen saturation was measured immediately using a calibrated GEM OPL instrument (Instrumentation laboratory, Bedford, MA) and haematocrit determined on arterialised samples using a Haematospin 1300 instrument (5 min, 4°C, Hawksley, Lancing, Sussex, UK) and nomogram.

### 5.3.3 Data Analysis

#### *Blood flow analysis*

During forearm plethysmography, 3 readings were taken at the beginning and end of each sampling period over a 10 minute period and the mean obtained. For adipose blood flow, Mediscint gamma-counter probes (Oakfield Instruments, Eynsham, UK) were used to record counts per second over 20 second intervals. The mean adipose flow was calculated over 30 minute intervals to correspond with the sampling periods.

#### *Kinetic analysis*

Whole body rate of appearance (Ra) of cortisol, Ra d3-cortisol and net Ra cortisone in the plasma were calculated by multiplying the rate of tracer infusion by the respective tracer:tracee ratio (*Equations 1-3*) (Stimson *et al*, 2009). Total body cortisone production was estimated using *Equation 4*.

#### *Equation 1:*

$$\text{Ra-cortisol (nmol/min)} = (\text{Rate of d4-cortisol infused} / \text{TTR(d4-cortisol: cortisol)}) - \text{Rate of cortisol infused}$$

#### *Equation 2:*

$$\text{Ra d3-cortisol} = \text{Rate of d4-cortisol infused} / \text{TTR (d4-cortisol:d3-cortisol)}$$

#### *Equation 3:*

$$\text{Net Ra cortisone (nmol/min)} = \text{rate of d2-cortisone infusion (nmol/min)} / \text{TTR (d2-cortisone:cortisone)}$$

Equation 4:

Total Ra cortisone (nmol/min) =

$$\frac{(\text{Rad3F (nmol/min)} / \text{concentration d3-cortisone (nmol/L)} + \text{net Ra-cortisone (nmol/min)})}{\text{Concentration d2-cortisone (nmol/L)}}$$

Subcutaneous adipose tissue and skeletal muscle production of cortisol, cortisone and d3-cortisol were calculated using arterialised (A) and venous (V) samples (superficial epigastric vein for adipose tissue, and deep forearm vein for forearm skeletal muscle) and blood flow over 2 steady state periods (period 1: 180- 210 min and period 2: 330-360 min) using the mean of 4 plasma samples for each time period. The following equations were used (Equations 5-8):

Equation 5:

Tissue cortisol release (pmol/100g tissue/min) =

$$\left( (\text{Blood flow} \cdot [\text{cortisol}]_{\text{artery}}) \cdot \left( \frac{\text{d4-cortisol:cortisol}_{\text{artery}}}{\text{d4-cortisol:cortisol}_{\text{vein}}} \right) - (\text{Blood flow} \cdot [\text{cortisol}]_{\text{artery}}) \right)$$

Equation 6:

Tissue d3-cortisol release (pmol/100g tissue/min) =

$$\left( (\text{Blood flow} \cdot [\text{d3-cortisol}]_{\text{artery}}) \cdot \left( \frac{\text{d4-cortisol:d3-cortisol}_{\text{artery}}}{\text{d4-cortisol:d3-cortisol}_{\text{vein}}} \right) - (\text{Blood flow} \cdot [\text{d3-cortisol}]_{\text{artery}}) \right)$$

Equation 7:

Net cortisone release from tissue (pmol/100g tissue/min) =

$$\left( (\text{Blood flow} \cdot [\text{cortisone}]_{\text{artery}}) \cdot \left( \frac{\text{d2-cortisone:cortisone}_{\text{artery}}}{\text{d2-cortisone:cortisone}_{\text{vein}}} \right) - (\text{Blood flow} \cdot [\text{cortisone}]_{\text{artery}}) \right)$$

Equation 8:

Total cortisone release (pmol/L/100g tissue/min) =

$((\text{Tissue d3-cortisol release} / [\text{d3-cortisol}]) \cdot [\text{d2-cortisone}]) + \text{net cortisone release}$

Data are presented as mean  $\pm$  SEM and were compared using paired Student's t-tests. Differences from zero were calculated using a single-sample t-test. Significance was taken at  $p < 0.05$ .

## 5.4 Results

### 5.4.1 LC-MS/MS assay optimisation

d2-Cortisone (mass +1,  $m/z$  364) gave a 3% signal which would interfere with d3-cortisone formed from the d4-cortisol tracer, if the two tracers were co-infused, d2-cortisol (mass +1,  $m/z$  366) gave a 1.6% signal and d2-cortisol (mass +2,  $m/z$  367) gave a 0.2% signal which would interfere with the d3-cortisol and d4-cortisol signals, respectively. It was determined that none of these background isotopic interferences contributed significantly as in plasma the signal did not exceed noise and did not need to be corrected for.

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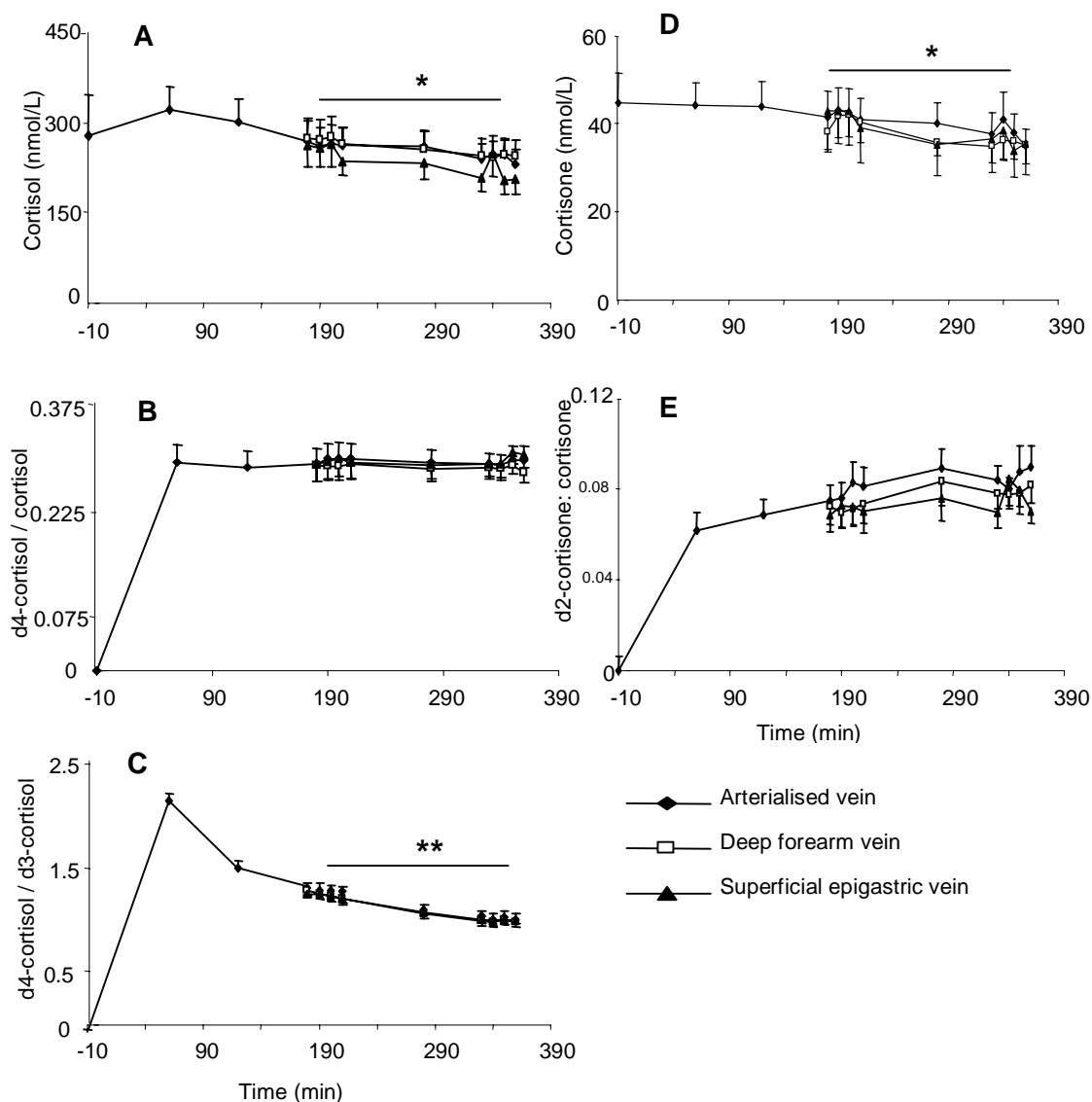
### 5.4.2 Whole body glucocorticoid metabolism

d4-Cortisol concentrations and enrichment of plasma with d4-cortisol were in steady state by 3 hours (Figure 5.2). d4-Cortisol enrichment did not change throughout the study (Table 5.1). There was evidence of whole body cortisol and d3-cortisol production (Table 5.2). During the hyperinsulinaemic clamp plasma insulin levels increased ( $9.9 \pm 0.9$  mmol/L prior

to insulin clamp vs  $83.4 \pm 4.0$  mmol/L during the insulin clamp,  $p < 0.0001$ ) but glucose levels remained stable ( $5.0 \pm 0.1$  mmol/L prior to insulin clamp vs  $5.1 \pm 0.1$  mmol/L during the insulin clamp,  $p = 0.69$ ). d4-Cortisol and cortisol concentrations fell, with a concomitant increase in plasma d3-cortisol concentration, rate of appearance of d3-cortisol, and fall in d4-cortisol:d3-cortisol ratio, consistent with increased  $11\beta$ -reductase activity (Table 5.1). Whole body production rates of cortisol did not change during hyperinsulinaemia (Table 5.2).

Plasma d2-cortisone concentrations and enrichment were in steady state at 3 hours and did not change during hyperinsulinaemia (Table 5.1). Dilution of d2-cortisone with cortisone was evident before insulin indicating  $11\beta$ -dehydrogenase activity (Table 5.2), but did not change during hyperinsulinaemia. Plasma concentrations of cortisone fell during hyperinsulinaemia (Table 5.1).





**Figure 5.2: Concentrations of endogenous glucocorticoids and tracer:tracee ratios measured in arterialised, deep forearm and superficial epigastric veins.**

Data are mean  $\pm$  SEM for  $n=6$  volunteers. The mean data for basal measurements were between  $t=+180-210$  minutes and for hyperinsulinaemia between  $t=+330-360$  minutes. (A) Cortisol concentrations; (B) d4-cortisol: cortisol ratio; (C) d4-cortisol:d3-cortisol ratio; (D) Cortisone concentrations; (E) d2-cortisone:cortisone ratios. Filled diamonds = arterialised vein; filled triangles = superficial epigastric vein; open squares = deep forearm vein; nM= nanomolar; d= deuterium; \* $p<0.05$  comparing arterial basal vs hyperinsulinaemia and \*\* $p<0.001$  comparing arterial basal vs hyperinsulinaemia using Student's paired t-tests.

### 5.4.3 Adipose tissue glucocorticoid metabolism

There were no arterio-venous differences in glucose, cortisol, d3-cortisol, d4-cortisol and cortisone concentrations (data not shown). In particular there was no arterio-venous differences in glucose during hyperinsulinaemia across the adipose tissue bed ( $5.1 \pm 0.1 \text{ mmol/L}$  arterialised vs  $5.1 \pm 0.1 \text{ mmol/L}$  venous adipose,  $p=0.57$ ), suggesting no additional uptake of glucose into adipose tissue during hyperinsulinaemia. However, a glucose tracer was not co-infused in this study which may have provided a more sensitive measure of tissue glucose uptake or release, as was used for tissue glucocorticoids.

Mean adipose tissue blood flow was unchanged during the study ( $5.7 \pm 1.3$  basal vs  $5.7 \pm 1.2$  ml/min/100g hyperinsulinaemia  $p=0.96$ ). Cortisol and d3-cortisol release, indicating reductase activity, were of a similar magnitude to previous studies (Stimson *et al*, 2009) but did not achieve statistical significance. Cortisone was released from subcutaneous adipose tissue (Table 5.2) consistent with adipose  $11\beta$ -dehydrogenase activity. Cortisol and d3-cortisol release did not change during hyperinsulinaemia. The fall in cortisone release during hyperinsulinaemia did not achieve statistical significance ( $n=5$ ), as one subject extracted cortisone across the adipose bed (Figure 5.3). The ratio of  $11\beta$ -reductase activity, as measured by Ra cortisol, to  $11\beta$ -dehydrogenase activity was  $\sim 1:1.5$ .

### 5.4.4 Skeletal muscle glucocorticoid metabolism

Mean forearm blood flow was unchanged during the study ( $2.8 \pm 0.2$  ml/min basally vs  $2.7 \pm 0.5$  ml/min,  $p=0.89$ ). There was significant release of cortisone, d3-cortisol and cortisol prior to hyperinsulinaemia indicating dehydrogenase and reductase activities respectively. During hyperinsulinaemia an arterio-venous difference in plasma glucose ( $5.1 \pm 0.1 \text{ mmol/L}$  arterialised vs  $4.3 \pm 0.3 \text{ mmol/L}$  venous forearm,  $p=0.035$ ) was measured indicating uptake of

glucose by the muscle bed. The ratio of  $11\beta$ -reductase activity to  $11\beta$ -dehydrogenase activity was ~1:1. Cortisone (Figure 5.3), cortisol and d3-cortisol release did not change during hyperinsulinaemia (Table 5.2).

**Table 5.1: Plasma steady state concentrations, ratios and blood flow during deuterated cortisol and cortisone infusions**

	Arterialised samples	
	Basal	Hyper-insulinaemia
C <sub>ss</sub> Cortisol (nmol/L)	263.8±30.6	241.5±25.4*
C <sub>ss</sub> d3-cortisol (nmol/L)	60.2±6.0	70.0±7.5*
C <sub>ss</sub> d4-cortisol (nmol/L)	76.3±6.9	67.9±5.3*
D4-cortisol: d3-cortisol	1.3±0.1	1.0±0.1**
D4-cortisol enrichment (%) (d4-cortisol/(cortisol +d4-cortisol))	22.8±1.3	22.6±1.0
C <sub>ss</sub> Cortisone (nmol/L)	42.0±5.3	37.9±4.7*
C <sub>ss</sub> d2-cortisone (nmol/L)	4.1±0.5	3.8±0.5
D2-cortisone enrichment (%) (d2-cortisone/(cortisone +d2-cortisone))	7.2±0.6	7.7±0.5
C <sub>ss</sub> Glucose (nmol/L)	5.0±0.1	5.1±0.1
Adipose tissue blood flow (ml/min/100g tissue)	5.7±1.3	5.7 ±1.2
Forearm blood flow (ml/min)	2.8±0.2	2.7±0.5

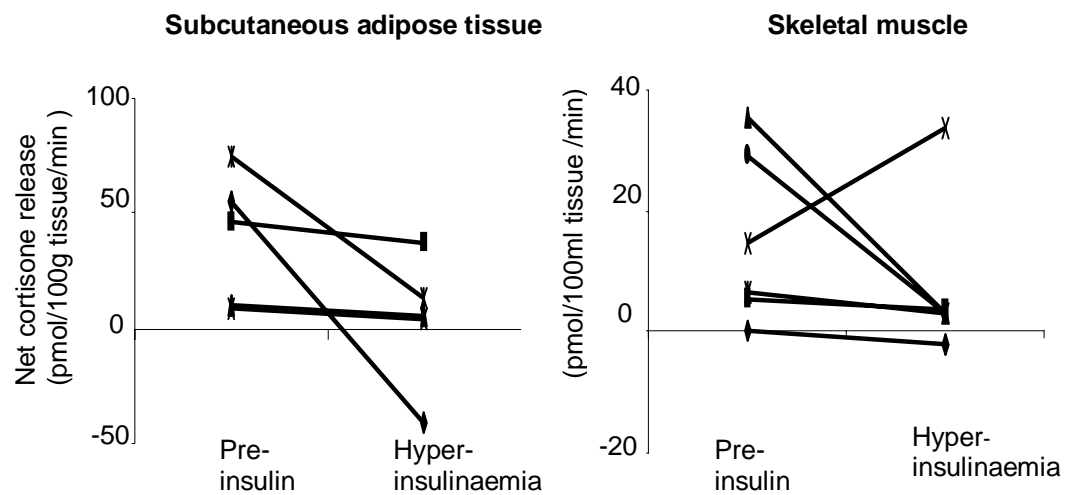
Data are mean ± SEM for n=6 volunteers. The mean data for basal measurements were between 180-210 minutes and hyperinsulinaemia between 330-360 minutes of tracer infusion. C<sub>ss</sub> = concentration at steady state; d=deuterium; \* p<0.05 comparing basal vs hyperinsulinaemia and \*\* p<0.001 comparing basal vs hyperinsulinaemia using Student's paired t-tests.

**Table 5.2: Calculated whole body 11 $\beta$ HSD1 and 11 $\beta$ HSD2 activity and cortisol and cortisone release across adipose tissue and skeletal muscle during deuterated cortisol and cortisone infusions.**

	Ra Cortisol		Ra D3-cortisol		Net Ra Cortisone		Total Ra Cortisone	
	Basal	Hyper- insulinaemia	Basal	Hyper- insulinaemia	Basal	Hyper- insulinaemia	Basal	Hyper- insulinaemia
Whole body (nmol/min)	62.6 $\pm$ 8.4†	63.0 $\pm$ 6.5	24.9 $\pm$ 1.0††	31.6 $\pm$ 1.6**	65.1 $\pm$ 6.9††	60.2 $\pm$ 5.6	72.49 $\pm$ 8.16††	70.07 $\pm$ 7.15
Adipose (pmol/100g tissue/min)	29.3 $\pm$ 21.1	20.8 $\pm$ 4.9	11.5 $\pm$ 7.9	12.4 $\pm$ 2.6	38.7 $\pm$ 13.0†	3.8 $\pm$ 12.6	44.56 $\pm$ 11.61†	9.28 $\pm$ 13.01
Muscle (pmol/100ml tissue /min)	19.7 $\pm$ 4.1†	16.5 $\pm$ 7.7	5.9 $\pm$ 1.8†	4.9 $\pm$ 1.5	15.2 $\pm$ 5.8†	7.5 $\pm$ 5.3	17.20 $\pm$ 5.94†	9.16 $\pm$ 5.30

Data are mean  $\pm$  SEM for n=6 volunteers. The mean data for basal measurements were calculated between 180-210 minutes and hyperinsulinaemia between 330-360 minutes of tracer infusion. Ra= rate of appearance; d=deuterium; \*\*= p<0.01 comparing arterial basal vs hyperinsulinaemic measurements using Student's paired t-tests; † = p<0.05 vs 0 and ††=p<0.001 vs 0 using single sample t-tests.

**Figure 5.3: Net rate of appearance of cortisone across subcutaneous adipose tissue and skeletal muscle beds for each study participant before and during hyperinsulinaemia.**



For subcutaneous adipose tissue: n=5 participants, for muscle: n=6 participants. One subject extracted cortisone across both adipose tissue and skeletal muscle.

## 5.5 Discussion

This chapter describes a study which used the stable isotope tracer d2-cortisone to quantify 11 $\beta$ HSD1-dehydrogenase activity *in vivo*. Substantial activity was present in both adipose tissue and forearm skeletal muscle of healthy men. Using d4-cortisol, significant forearm skeletal muscle 11 $\beta$ HSD1-reductase activity was also demonstrated. The subcutaneous adipose tissue 11 $\beta$ HSD1-reductase results are consistent with the previous report quantifying subcutaneous adipose 11 $\beta$ HSD1-reductase activity (Stimson *et al*, 2009). The study data also confirm the finding that hyperinsulinaemia increases whole body 11 $\beta$ HSD1-reductase activity (Wake *et al*, 2006) *in vivo*. Furthermore, the ratio of dehydrogenase to reductase activity is ~1 in the whole body and skeletal muscle and ~1.5 in subcutaneous adipose tissue suggesting net inactivation of cortisol in adipose tissue.

Intracellular glucocorticoid cycling may be the mechanism by which cells control the local intracellular environment to enhance or dampen the effects of glucocorticoids in specific tissues rather than simply reflecting circadian or ultradian changes in circulating glucocorticoid levels. Some primary cell cultures, eg adipose stromal vascular cells and Leydig cells, display both 11 $\beta$ HSD1 reductase and dehydrogenase activity (Bujalska *et al*, 2002;Gao *et al*, 1997) permitting glucocorticoid cycling in these tissues. It has been postulated that these activities regulate the balance between cellular proliferation and differentiation and protect normal physiological hormone secretory patterns (Bujalska *et al*, 2002;Gao *et al*, 1997). The finding of both reductase and dehydrogenase activities in whole subcutaneous adipose tissue demonstrates that both these activities occur *in vivo* too, and may have a similar function in whole tissue and cells, although this study was not designed to explore the function of these activities.

In some cells and tissues, eg hepatocytes / liver,  $11\beta$ -reductase activity prevails *in vitro* (Jamieson *et al*, 1995) and *in vivo* (Rask *et al*, 2001). Whether substantial glucocorticoid cycling occurs in the liver is not yet known. Using stable isotope tracers our group have been unable to measure cortisone production in the hepatic vein, but this probably reflects low plasma cortisone concentrations and inadequate instrument sensitivity to detect these levels rather than complete absence of dehydrogenase activity.

Both net and total cortisone production have been presented as the d2-cortisone tracer may be recycled *in vivo* by  $11\beta$ -dehydrogenase. By co-infusing d2-cortisone with d4-cortisol this recycling can be estimated through measuring d3-cortisone and d3-cortisol interconversion. This assumes a linear relationship of recycling rate with cortisol and cortisone concentrations and steady state with dehydrogenase and reductase activities for recycling. As d4-cortisol is not regenerated from d3-cortisol, recycling by  $11\beta$ HSD1-reductase does not need to be accounted for with d4-cortisol. Whole body net cortisone production rate was increased by 8-10nmol/min and tissue production between 2-6pmol/min with this calculation.

Confirming previous *ex-vivo* reports (Bujalska *et al*, 2002) the source of dehydrogenase activity in adipose tissue is likely to be  $11\beta$ HSD1, as adipose tissue  $11\beta$ HSD2 expression has been reported as negligible (Bujalska *et al*, 2002) or much lower than that of  $11\beta$ HSD1 in subcutaneous adipocytes of obese women (Engeli *et al*, 2004), and the stromal fraction of human omental adipose tissue (Lee *et al*, 2008). Also techniques which may cause dissociation of  $11\beta$ HSD1 from the co-localized H6PDH, eg micro-dialysis (Wake *et al*, 2006) were not used in this study. In mice, increased adipose tissue dehydrogenase activity reduces the adverse metabolic effects of high fat feeding when  $11\beta$ HSD2 is over-expressed in adipocytes (Kershaw *et al*, 2005). Thus, adipose tissue  $11\beta$ HSD1-dehydrogenase activity may be metabolically beneficial in man. The origin of dehydrogenase activity in forearm samples is less clear cut as  $11\beta$ HSD2 is expressed in skeletal muscle vascular cells,



interstitial cells and perhaps in the myocyte (Jang *et al*, 2007). Although other investigators have not replicated these findings in needle biopsy muscle samples (Whorwood *et al*, 2001; Abdallah *et al*, 2005). Other potential sources of dehydrogenase activity in the forearm samples include the vasculature (Christy *et al*, 2003), bone, skin and adipose. As 11 $\beta$ HSD2 expression is low in non-fetal osteoblasts (Cooper *et al*, 2000), negligible in adipose tissue (Bujalska *et al*, 2002), not expressed in the epidermis (Hirasawa *et al*, 1997), and blood flow from the hand was excluded in the study, it is likely that forearm skeletal muscle does display significant 11 $\beta$ HSD1-dehydrogenase activity *in vivo*.

Previous *in vivo* studies measuring arteriovenous gradients in cortisol and cortisone concentrations, using standard forearm arteriovenous sampling or incremental intra-arterial cortisol infusions, failed to demonstrate forearm 11 $\beta$ HSD1-reductase activity (van Uum *et al*, 2002; Katz *et al*, 1999; Asmal *et al*, 1974) despite skeletal muscle 11 $\beta$ HSD1 expression (Jang *et al*, 2006). However, stable isotope tracers and mass spectrometry were not used in these studies, which may have reduced the precision of the measurements especially if, as our data suggests, there is cycling of cortisol and cortisone. Using d4-cortisol, 11 $\beta$ -reductase activity has been demonstrated in the leg of obese subjects (Basu *et al*, 2004; Basu *et al*, 2005), but as in this study, other tissues such as skin, vasculature and bone may have contributed to this activity. Furthermore, indocyanine green was used to measure blood flow which may not be significantly extracted across the leg, and venous flow from the foot was not excluded (Basu *et al*, 2004; Basu *et al*, 2005). Using different blood flow methods and skeletal muscle depot this study shows that there is significant reductase activity in human skeletal muscle of healthy subjects.

To calculate the ratio of 11 $\beta$ -dehydrogenase to reductase activity rate of appearance (Ra) cortisol:cortisone was employed. Although Ra d3-cortisol is more specific for 11 $\beta$ HSD1-

reductase activity than Ra cortisol, the substrate, d3-cortisone, may not have been in steady state in the tissues, compared to endogenous cortisol at the time of sampling leading to an under-estimation of Ra. *Ex-vivo* (Bleau *et al*, 1974) and *in vivo* studies (please see data from Chapter 3) have demonstrated the slow uptake of glucocorticoids into adipose tissue, and thus it is unlikely that changes in adrenal cortisol secretion will have been reflected in adipose in this study and will not have contributed to the Ra cortisol obtained from arteriovenous sampling of tissues.

In non-fasted obese subjects receiving an intravenous infusion of dextrose, subcutaneous adipose tissue 11 $\beta$ -reductase activity has been quantified as 15 and 8.7 pmol/min/100g adipose for cortisol and d3-cortisol respectively (Stimson *et al*, 2009). Our results in lean fasted men are of a similar magnitude to this but did not achieve statistical significance as in one subject both cortisol and d3-cortisol were extracted across the adipose tissue bed.

The data from this study suggest that local production and turnover of cortisol and cortisone are lower per volume of tissue in skeletal muscle than in subcutaneous adipose tissue. Physiologically this higher rate of local cortisol production in adipose tissue may be a mechanism to selectively increase adipose tissue lipolysis and release fuel for gluconeogenesis, whilst relatively sparing skeletal muscle from proteolysis during times of caloric restriction, such as fasting during this study, thereby preserving muscular integrity. In skeletal muscle, short courses of oral glucocorticoids have detrimental effects on muscle insulin sensitivity which reverse upon cessation of therapy (Short *et al*, 2009). Thus lower local cortisol production in skeletal muscle may also be a mechanism to preserve muscle insulin-glucose homeostasis (Morgan *et al*, 2009).

The effects of the non-selective 11 $\beta$ HSD inhibitor carbenoxolone have been examined in rodent and human studies producing variable results. In Zucker rats, carbenoxolone had no

effect on fasting glucose levels or 11 $\beta$ HSD1 activity in skeletal muscle or adipose tissue, but did inhibit 11 $\beta$ HSD1 activity in the liver (Livingstone & Walker, 2003). When administered to healthy lean men carbenoxolone improved whole body insulin sensitivity, probably through suppression of hepatic glucose production, but had no effect on forearm insulin sensitivity (Walker *et al*, 1995). As skeletal muscle has both 11 $\beta$ HSD1 reductase and dehydrogenase activities and glucocorticoid production in forearm tissue appears relatively slow, the absence of an effect of carbenoxolone in this tissue may be due to both the non-selective nature of carbenoxolone (blocking both activities) and the slow tissue production. Furthermore, carbenoxolone undergoes hepatic first pass metabolism and is highly concentrated in the liver, suggesting that low extra-hepatic concentrations of drug may poorly penetrate other tissues and may explain the variable response to carbenoxolone in the published literature.

Altered endoplasmic reticulum NADPH:NADP<sup>+</sup> ratio is believed to change 11 $\beta$ HSD1 directionality because reduced local supply of NADPH in H6PDH null mice alters the direction of 11 $\beta$ HSD1 from reductase to dehydrogenase (Lavery *et al*, 2006). As glucose-6-phosphate (G6P) is a substrate for H6PDH and intracellular G6P levels may be dependent on glucose, we hypothesised that hyperinsulinaemia may increase glucose flux through the pentose phosphate pathway, leading to an increase in NADPH within the ER, which may in turn push tissue 11 $\beta$ HSD1 activity towards reductase *in vivo*. Hyper-insulinaemia increased whole body d3-cortisol production as previously (Wake *et al*, 2006), but had no significant effect on tissue 11 $\beta$ -reductase activity or blood flow. However, arteriovenous differences in plasma glucose, suggesting flux of glucose into tissues, were only seen in plasma samples from the forearm and not in plasma draining adipose tissue. Thus hyperinsulinaemia may not have increased glucose flux into adipose tissue and this may be a potential reason for the lack of change in 11 $\beta$ HSD1 activity in adipose tissue. Furthermore, activity of the pentose phosphate pathway is low in muscle compared to tissues such as the liver and adrenal. This

may further explain why despite an arterio-venous difference in glucose in forearm plasma samples, a difference in tissue 11 $\beta$ HSD1 activity was not seen.

Adipose tissue cortisone production tended to fall with hyperinsulinaemia, but again in one subject cortisone was extracted across the adipose bed and the study was not of sufficient power to detect whether this change was real or not. Therefore, although the results suggest that hyperinsulinaemia does not affect 11 $\beta$ HSD1 directionality in tissues, this will need to be tested in a larger number of subjects in further studies.

Using DXA-derived body fat measurements to assess total body adipose tissue mass along with adipose glucocorticoid release figures, I have extrapolated that adipose 11 $\beta$ HSD1 contributes 7.3 nmoles /min of cortisone, which is ~10% of whole body cortisone production rate calculated here. This figure is based on the assumption that the 11 $\beta$ HSD1 substrate (d2-cortisone) was in steady state during the measurements, and 11 $\beta$ HSD1 expression and activity were uniform during the study. During the study mean blood flow in the subcutaneous adipose tissue depot was confirmed to be stable, but biopsies of adipose depots were not collected for assessment of 11 $\beta$ HSD1 expression, nor factors such as prolonged fasting assessed when measuring adipose blood flow (Frayn *et al*, 2003) and thus this figure needs to be interpreted within these limitations. The contribution of adipose cortisol production to whole body rates was marginally lower than previously reported at 10.2% (vs 12 %) (Stimson *et al*, 2009). These differences may relate to our subjects being leaner and having lower adipose tissue 11 $\beta$ HSD1 activity, as measurements in adipose biopsies suggest that activity is ~3-fold higher (per gram adipose tissue) in obesity (BMI 31.7kg/m<sup>2</sup>) compared to normal weight (BMI 22.9kg/m<sup>2</sup>) (Rask *et al*, 2001).

In conclusion, both 11 $\beta$ HSD1 reductase and dehydrogenase activities occur *in vivo* in human forearm skeletal muscle and subcutaneous adipose tissue suggesting that cycling of cortisol

and cortisone at the tissue level may occur. Whole body  $11\beta$ HSD1 reductase activity was increased by hyperinsulinaemia but activity and directionality do not appear to be influenced by hyperinsulinaemia in tissues.

## **Chapter 6**

## **Conclusions**

Obesity is an increasing problem in society with almost a quarter of adults in the UK being classed as obese. Obesity is associated with an increased adipose tissue mass which has the ability to generate hormones and cytokines with local and systemic effects. Adipose tissue expresses the enzyme 11 $\beta$ HSD1 which has previously been thought to predominantly generate active glucocorticoid from inert cortisone in tissues. Mice over-expressing 11 $\beta$ HSD1 in adipose tissue develop a metabolic syndrome phenotype (Masuzaki *et al*, 2001). In human obesity subcutaneous adipose 11 $\beta$ HSD1 is also up-regulated (Rask *et al*, 2001). Since the increase in 11 $\beta$ HSD1 in adipose tissue in these mice is similar to the up-regulation observed in adipose tissue in human obesity, these findings suggest that increased intra-adipose 11 $\beta$ HSD1 may be responsible for the metabolic complications of obesity and has led to this enzyme becoming a therapeutic target. However many important questions regarding adipose tissue 11 $\beta$ HSD1 remain and this thesis aimed to address some of them.

After establishing a method to extract and measure adipose tissue glucocorticoids, the results described in Chapter 3 helped address the issue of absolute adipose tissue glucocorticoid levels, uptake of glucocorticoids into adipose tissue and the contribution of 11 $\beta$ HSD1 to the intra-adipose glucocorticoid pool. Adipose tissue glucocorticoid levels were higher than previously reported using a LC-MS/MS assay and this may have reflected the assay development strategy to remove the steroids from the tissue completely rather than analyse the crude tissue extract.

*In vivo* data suggested that accumulation of glucocorticoids into adipose tissue is slow with only ~10% of the intra-adipose glucocorticoid pool replaced per hour. To my knowledge no previous *in vivo* studies have studied cortisol transport and turnover in adipose tissue, although one *ex-vivo* study reported adipose uptake of [<sup>3</sup>H]-cortisol was ~5 times slower than that of sex steroids and androgens (only 12.7% uptake at 3 hours) (Bleau *et al*, 1974). In the

rat brain, ultradian pulses of glucocorticoids are quickly reflected in hippocampal extracellular fluid in rodents (Droste *et al*, 2008) and *in vitro* induce the pulsatile transcription of RNA from GR-regulated genes (Stavreva *et al*, 2009) suggesting uptake in the rodent brain and *in vitro* is quicker than adipose tissue and that there may be a rapid signalling system in place to effect these changes. The results in Chapter 3 suggest that this may not be the case in adipose tissue and the intra-adipose cortisol pool may have a role in integrating the longer-term variations in circulating glucocorticoid concentrations compared to the brain and liver. Moreover, unless there are multiple pools of glucocorticoid within adipose tissue, with a cytosolic ‘free’ pool that turns over more quickly than a triglyceride-bound pool, then it appears unlikely that intra-adipose cortisol concentrations vary widely during acute changes in plasma cortisol, but this delayed rate needs to be confirmed under normal physiological circumstances where adipose blood flow, meal pattern and insulin levels normally occur, rather than during surgery.

The rates of regeneration of cortisol by intra-adipose 11 $\beta$ HSD1 measured in previous studies (Stimson *et al*, 2009) were high compared with the rate of accumulation of plasma-derived cortisol within adipose, emphasising the importance of 11 $\beta$ HSD1 in human adipose tissue glucocorticoid signalling. It will be important to establish the rates of exchange between plasma and other intra-cellular cortisol pools in other organs. Indeed, further studies assessing time to steady state of d4-cortisol in adipose tissue of rodents and assessing adipose glucocorticoid transporters are underway. These may further elucidate the mechanisms of glucocorticoid transport into adipose tissue and may help identify why glucocorticoid uptake in adipose tissue appears slow.

To address the question as to whether adipose tissue 11 $\beta$ HSD1 displays dehydrogenase activity *in vivo*, a new stable isotope tracer technique was developed to measure 11 $\beta$ -



dehydrogenase activity *in vivo* as described in Chapter 4. After optimising tracer measurement using LC-MS/MS and establishing that it was a substrate for human 11 $\beta$ HSD1, a primary isotope effect was also excluded. Pharmacokinetics were derived *in vivo* and whole body net cortisone production rate measured before and after eating liquorice which contains inhibitors of 11 $\beta$ -dehydrogenase and a substantial fall in whole body cortisone production was observed.

Using arterio-venous sampling techniques and the expertise of staff in the Clinical Research Facility, OCDEM, University of Oxford, Oxford, subcutaneous adipose tissue and skeletal muscle 11 $\beta$ HSD1 reductase and dehydrogenase activities were simultaneously measured. The findings reported in Chapter 5 demonstrate that 11 $\beta$ HSD1 is bidirectional in human subcutaneous adipose tissue and skeletal muscle *in vivo*, suggesting that glucocorticoids can cycle in these tissues. Intracellular glucocorticoid cycling may be the mechanism by which cells control the local intracellular environment to enhance or dampen the effects of glucocorticoids in specific tissues rather than simply reflecting circadian or ultradian changes in circulating glucocorticoid levels. Some cells, eg adipose stromal vascular cells and Leydig cells, display both 11 $\beta$ HSD1 reductase and dehydrogenase activity (Bujalska *et al*, 2002; Gao *et al*, 1997) permitting glucocorticoid cycling in these tissues. Whether the impact of this glucocorticoid cycling is autocrine or paracrine is not known.

The finding that 11 $\beta$ HSD1 is bidirectional in metabolically important tissues leads to the question as to what will happen to this activity when transcript levels are increased eg in subcutaneous adipose tissue in obesity. To study this one could repeat the study described in Chapter 5 with obese individuals and also take subcutaneous adipose tissue biopsies to confirm the increased 11 $\beta$ HSD1 mRNA in the adipose of these subjects. If both 11 $\beta$ HSD1 reductase and dehydrogenase activities increased in parallel, glucocorticoid balance in the

tissue will not be disrupted, but if this equilibrium was disturbed due to other as yet undiscovered factors affecting 11 $\beta$ HSD1 activity and directionality this may be metabolically detrimental. From a physiological perspective, an increase in both dehydrogenase and reductase activities appears futile, but may have a role to buffer any compensatory changes in cortisol concentrations.

Increasing glucose flux into tissues and thus increasing NADPH co-factor supply does not appear to regulate the balance of activities of 11 $\beta$ HSD1 in adipose tissue or skeletal muscle. However, we were only able to demonstrate an increase in uptake of glucose into skeletal muscle only. The study assessing 11 $\beta$ HSD1 during hyperinsulinaemia was not powered to detect these changes and studying additional healthy individuals may help answer the question further. Nevertheless it can be concluded from this study that the dysregulation of 11 $\beta$ HSD1 transcription in obesity in adipose tissue may affect cortisol inactivation as well as regeneration.

To conclude, these studies confirm that 11 $\beta$ HSD1 has an important role in adipose tissue glucocorticoid homeostasis. Traditional teaching regarding glucocorticoid action in adipose tissue suggests that cortisone and cortisol enter the cell, that an additional source of intracellular cortisol is reduction of cortisone by 11 $\beta$ HSD1, and this cortisol can activate adipose glucocorticoid receptors, or be metabolised by 5 $\alpha$ -reductase. However, the results from these studies suggest that in addition to the above model, although these findings need to be confirmed under physiological conditions, we can now add that transport of glucocorticoids into adipose tissue is slow and we speculate that there are 2 cortisol pools in adipose tissue – one free pool able to interact with glucocorticoid receptors rapidly and a further triglyceride-bound pool, which probably takes a longer period of time to ‘fill’ and equilibrate. Furthermore, the studies suggest that in adipose tissue an additional source of

both cortisol and cortisone results from cycling of glucocorticoids. Whether this cycling is autocrine or occurs in nearby stromal vascular cells resulting in a paracrine effect is not known.

Through developing new techniques to measure 11 $\beta$ HSD1 in tissue samples and *in vivo* we have furthered the understanding of normal adipose 11 $\beta$ HSD1 physiology. These techniques may in turn be applied to further studies in obesity where adipose 11 $\beta$ HSD1 is dysregulated. Furthermore, the work described in this thesis suggests that 11 $\beta$ HSD1-inhibitors do need to be selective for 11 $\beta$ HSD1-reductase to ensure they give the maximum metabolic benefits from inhibiting adipose tissue 11 $\beta$ HSD1.

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